

A Literature Review on Quantitative Assays in Viral Immunology

Jared Galloway — Fred Hutchinson CRC

November 29, 2020

Abstract

Protection from rapidly spreading and evolving viruses is key to human health and survival. The molecular nature of infection and respective immune system defences presents us with a complex and noisy set of problems to solve if we are to combat infectious disease and understand the nature of their evolution. Most notably, we need to understand the affinity of a virus to bind to a host cell via proteins expressed on the surface of the cell's outer membrane. These proteins allow the virus to enter a host cell, then proceed to hijack the cell's own machinery to replicate and propagate an infection within the host individual. In the context of SARS-CoV-2, understanding the immune response with respect to those binding proteins is critical for prevention and prediction of disease severity. Additionally, understanding the evolution of these binding sites allows us to interpret active selection among the viral population as well as duration of host immunity post-infection. Neither of these are trivial problems to solve. Fortunately, recent advances in next generation sequencing (NGS), oligonucleotide synthesis (ONS), and PCR-induced mutagenesis have driven the development of quantitative assays and given us the ability to explore and quantify fitness of particular proteins in the context of binding affinity to entry sites on host cells. These methods have laid the foundation for rapid exploration and development of advanced vaccines which provide protection against deadly pathogens. In this literature review, we explore two techniques which leverage *quantitative assays*; Phage Immunoprecipitation Sequencing (PhIP-Seq) and Deep Mutational Scanning (DMS). In short, these methods allow us to measure and explore binding properties of relevant proteins and thus provide insight into the nature of a virus with respect to its *pathogenesis*. Concretely, we will provide background and motivation of quantitative assays by observing the results and methods from Shrock et al. [2020] and Starr et al. [2020] – two studies which focus on the binding properties of the novel betacoronavirus, SARS-CoV-2 and its potential variants. The primary motivation behind this literature review is to provide an approachable explanation of novel techniques which are being used to rapidly characterize how we are combating novel, viral outbreaks.

Primer & Prologue *You are welcome to skip this section if you're well versed the history and relevance of the Central Dogma and Basic Viral Biology. For me, priming myself with the basics before diving deep into complex topics like the ones described in this paper is always beneficial.*

Every organism on earth is the aggregate of many underlying biological systems which work together to drive the complex functions which fully characterize every individual. In more detail, each biological system is driven by a defined set of proteins – each encoded and regulated by a relatively small portion the individual's genetic code. Each protein has a role to play which may be trivial by itself, but in concert with the other relevant proteins we observe incredible functionality. The advent of Next Generation Sequencing (NGS) and complimentary algorithms has provided researchers with the ability to explore entire genome sequences from almost any living organism of interest. Even more impressively, we can profile these protein coding portions of the genome by extracting messenger ribonucleic acid (mRNA) from cells to infer the set of all expressed proteins, known as the *proteome*. In contrast to living organisms, the genetic code of a virus is extremely simplistic. In the case of RNA viruses such as SARS-CoV-2, genomes are consisted mostly of protein coding RNA with a limited set of instructions. With no means to replicate or produce energy alone, the primary function of viral proteins is to bind to complimentary proteins expressed on host cells and hijack the machinery in order to reproduce itself - a process which then destroys the cell. Unfortunately little is known about how the underlying peptide sequences and relates to its folding properties (tertiary structure) and most importantly, its primary function. Exploring the function of proteins and their properties is a necessity when uncovering the labyrinth of biological systems and developing therapeutics to combat disease.

Introduction

Modern mammalian immune systems are constituted by the aggregate of proteins and specialized cells known as *lymphocytes* which defend against unwanted invaders (pathogens). These defences keep the pathogens from harming the delicate and complex biological systems which keep us alive and healthy. However, deadly pathogenic outbreaks which rapidly spread among humans and other species can often harm or kill a large percentage of populations [Wu et al., 2020]. In the case of viruses, replication as a function of fitness drives pathogens to evolve much in the same way we do – often meaning the most potent and infectious pathogens prevail as a product of their genome evolution [Twiddy et al., 2003, Felsenstein, 1981]. Fighting fire with fire, the adaptive immune system works through similar processes of mutation and selection inside our own body to evolve along-side these pathogens and confer specialized protection against pathogens. In many cases, this protection from a virus lasts throughout the lifetime of an individual. Incredibly, the combinatorial effects of specialized (VDJ) recombination results in enough diversity to select upon that evolution of specialized cells takes place in mere days (often a week or so) when encountering a new pathogen [Jung and Alt, 2004].

In contrast to all other forms of evolution (often on ecological timescales), The process of generating specific antibodies to ward off an infection is incredibly fast. Unfortunately, the symptoms of an infection during that time frame can still make an individual very ill, or even be fatal. The ability of viruses to replicate itself in order to propagate the infection make them efficient and deadly. Luckily, the process of producing antibodies need not occur every time we encounter the same virus. Rather, once an individual has encountered a pathogen and created the necessary cells to fend off the virus and infected cells the defences that were created are stored in a sort of “immuno-memory” – using another type specialized cell. Upon contact with a pathogen the individual has encountered in the past, then, the immune system has the infrastructure in place to elicit a fast and effective response. Having this cellular machinery is what’s known as *immunity* in an individual - and is key to survival in a world filled with microbial pathogens.

One of the most impactful developments in human health has been our ability to provoke immunity to common viruses without actually infecting us with a deadly disease causing pathogen. These biologically prepared agents are known as *vaccines* – and according to the center for disease control (CDC.gov) will have prevented over 21,000,000 hospitalizations and roughly 750,000 deaths among children born within the last 20 years in the U.S alone. While this is an extreme success, the rate at which vaccines can be produced are a function of our ability to observe the physical properties of a virus. To date, the fastest a vaccine that has been successfully developed was during the mumps outbreak in 1969 and took 4 years from start to finish. Facing a more deadly pathogen, this slow rate of development could pose an existential threat to modern populations in a globalized world.

Commonly, a vaccine for some particular virus essentially models the virus without any of the harmful properties. This can be thought of as giving your immune system a molecular picture of the virus so that it is prepared when the real thing is encountered. Any molecular sequence that elicits an immune response is known as an *antigen* – and the antigen targeted by antibodies for a particular set of pathogens is known as the *epitope*. Inferring the epitope for any virus is key for developing vaccines and stands as tough problem in the case of most viruses. To date there is no direct way to isolate which proteins are expressed on a virus, and which constitute an antigen. The number of possible proteins which could constitute an epitope for any particular virus is defined by every possible sub-sequence within its respective proteome. To complicate further, little is known about how sequence variation from mutation impacts protein function as the pathogen evolves. We would like to know how possible mutations impact our immuno-defences; in the case of the novel coronavirus, SARS-CoV-2, high mutation rates have already been found in the region which binds to our cells [Stoddard et al., 2020]. In order to predict or understand how long immunity will last in the face of evolution, we must explore all variants of the epitope and their respective binding affinity relative to the wild type sequence.

Fortunately, recent advances in techniques such as next generation sequencing (NGS), oligonucleotide synthesis (ONS), modern computing and more, have opened the door to a new brute force, high throughput approach to exploring these outstanding questions. The conjunction of these advancements have provided the infrastructure necessary to create and analyze *Quantitative assays* in immunology. Using the related protocols and analysis techniques, researchers now have the ability to explore the relevant likelihoods of nearly every single possible antigen – as well as every possible mutant of a particular antigen. These large scale studies require complex and carefully executed protocols resulting in large and noisy data for analysis [Mohan et al., 2018, Thyagarajan and Bloom, 2014, Shrock et al., 2020]. Once the data is acquired, advanced modeling and computing techniques are applied to parse the signal from noise and produce some form of likelihood surrounding a particular sequence.

This likelihood then informs a great deal about the biological nature of an evolutionary tango between pathogens and an individual's immune system [Garrett et al., 2020]. Quantitative assays in Immunology, particularly in the last decade, have laid the foundation for measuring protein interactions between a virus and individual host cells at a magnitude far greater than was previously possible with traditional techniques [Fowler and Fields, 2014, Thyagarajan and Bloom, 2014].

Here, we dig into the benefits and limitations of two such quantitative assays, Phage Immunoprecipitation Sequencing (PhIP-Seq), and Deep Mutational Scanning (DMS). We will explore the methods, results, and analysis tools of two studies using these assays applied to proteins expressed by SARS-CoV-2. These studies will act as a template for understanding the biological insight provided by quantitative assays as a whole. First, we explore Shrock et al. [2020], a large-scale PhIP-Seq study done at Harvard university to characterize the set of epitopes found in SARS-CoV-2. Next, we observe how every single mutation across the protein responsible for viral entry on the novel coronavirus impacts the affinity of binding to ACE2 receptor on human cells in Starr et al. [2020]. Together, these studies motivate the use of quantitative assays and associated analysis techniques in Immunology.

Quantitative Assays in the Context of SARS-CoV-2

The RNA genome of SARS-CoV-2 consists of $\approx 30,000$ nucleotides which provide the landscape for 11 protein coding regions [Naqvi et al., 2020]. Of those, there exists a very small subset of less than 90 nucleotides encoding for epitopes identifying the virus to lymphocytes and invoking an immune response able to combat the infection. Designing effective therapeutics and understanding how their effectiveness as the virus evolves over time provokes two primary questions (among many others): (1) Given the viral genome, how can we accurately infer the correct epitopes which will act as the appropriate antigen and (2) how effective will the chosen antigenic sequence be in the face of inevitable viral genome evolution of SARS-CoV-2. In this section we explore two recent studies which leverage commonly used quantitative assay techniques in the field of viral immunology to explore these specific questions. For each of these quantitative assay methods, we describe the methods and their respective history, followed by the primary results of two novel studies.

Phage Immunoprecipitation Sequencing (PhIP-Seq)

Addressing the question of inferring the profile of virus epitopes targeted by individual antibodies, we turn to one of the more notable quantitative assay techniques, known as Phage Immunoprecipitation Sequencing (PhIP-Seq). Originally, Larman et al. [2011] introduced this method which used oligonucleotide synthesis to create a library representation of all possible proteins generated by the human proteome. This quantitative assay was then used to identify which human proteins were being mislabeled as pathogens and attacked by the individual's own antibodies – thus leading to auto-immune diseases such as Diabetes type I, multiple sclerosis, and rheumatoid arthritis. These synthetic protein libraries are commonly referred to as *peptidomes* [Mohan et al., 2018]. The entire span of possible proteins that may be expressed by a proteome of interest is created using a sliding window approach. The sliding window begins at the start codon and spans some number of nucleotide triplets to encode for a potential protein. The window then jumps some number of codons down the sequence to create an adjacent (and usually overlapping) protein encoding sequence. This process continues throughout an open reading frame (ORF) until all possible protein encoding sub-sequences of oligonucleotides are included in the library. It should be noted that the chosen size of the window, or “tile” – and how much overlap each tile shares with adjacent tiles – will control the granularity of the assay and thus the detail of the resulting data. This is a direct trade-off between the size and cost of the synthetic library and the molecular detail of your results. Once the library is designed computationally, oligonucleotide synthesis is used to generate all genetic “tiles” encoding proteins of interest in the library. The synthetic oligonucleotide encodings are then cloned into a phage vector display (usually T7 phage). Once cloned, the protein coding nucleotides of interest are then transcribed using the machinery of the phage and expressed on the exterior to create a peptidome library.

The overarching concept of this technique is to create and label (barcode) a set of proteins of interest - this is the quantitative assay. Once developed, this assay is then presented (*homogenized*) with serum antibodies extracted from a patient of interest. Finally, the binding interactions between an antibody and specific protein are extracted (precipitated) using specialized magnetic beads. The resulting binding events give us a set of barcoded genetic information within phage which are extracted by a lysis step then sequenced using NGS. When

the demultiplexed samples are aligned with the library of oligonucleotides, we can count the number of each barcoded peptide which had a binding event with the antibodies of a sample. The resulting data from this process is then a counts array where each count represents the number of binding events for each of the peptides in the library. When repeating this process with multiple samples, the arrays for each sample are merged to create what is most commonly referred to as the *enrichment matrix*. It then follows that large numbers in the counts matrix represent some underlying binding affinity of a particular antibody to a specific peptide. With respect to epitope detection in viruses of interest, this same technique is applied using the proteome of the virus to create the peptidome. We can then explore which proteins are targeted by antibodies produced during the infection of some patient or individual. To summarize, this results in a picture of exactly which protein sequences an individual’s antibodies are targeting. Given that the antibodies were generated as a direct response to a viral infection, we can expect the binding profiles to reveal all potential epitopes across a viral proteome.

The first application of PhIP-Seq for viral epitope profiling was presented in Xu et al. [2015], where the authors developed a peptidome library encapsulating all known human-infecting viruses – the *Virscan* library. Virscan contains over 100,000 proteins and offers the most broad view of potential epitopes to date. Since the introduction of this technique in immunology, a multitude of libraries for viral epitopes have been created and used in subsequent studies to identify detailed maps of individual antibody binding repertoires. Next, we explore how PhIP-Seq has been used to profile the antibody binding affinities and potential epitopes of SARS-CoV-2.

Peptidomes to profile SARS-CoV-2 epitopes In Shrock et al. [2020], the immune (humoral) response of a mixed cohort of both COVID-19 positive and pre-pandemic individuals (232 and 190, respectively) was quantified by presenting IgG and IgA antibody (*serological*) samples to a set of synthetic peptidomes using PhIP-Seq. This large scale study provided insight into many outstanding questions surrounding differential immune response to COVID-19. In addition to the Virscan peptidome described above, the authors present three additional synthetic peptidomes, each to provide a different scope and granularity of candidate epitopes across viral proteomes of interest. One of the libraries focused specifically on including proteins from all human-infecting coronaviruses (HCoV’s). Among the HCoV’s, this included four endemic coronaviruses which cause the common cold including OC43, HKU1, NL63, and 229E and three severe acute respiratory disease causing HCoV’s; SARS-CoV, MERS, and SARS-CoV-2. The HCoV library encapsulated all protein coding regions from the viral genomes with a 56-mer amino acid (aa) window, tiling every 28aa. For a more detailed look at SARS-CoV-2, the authors presented a library across the virus with 20-mer aa windows across the viral proteome tiling every 5aa. Finally, the authors produce a library across the SARS-CoV-2 proteome with 56-mer aa windows tiling at every codon, except at each location they introduce a triple alanine mutation to precisely define epitope boundaries. In total across all samples and libraries, this study measured more than 10^8 unique peptide-antibody binding interactions. Using these individual profiles, the authors explore questions about COVID-19 disease outcome and SARS-CoV-2 epitopes which may be shared with other common human coronaviruses (HCoV’s).

SARS-CoV-2 Epitopes and cross-reactivity with endemic HCoV’s The authors analyze the enrichment matrix looking for significant enrichments among background noise by using a z-score approach and assigning p-values to each of the enrichments. The subset of significant enrichments among the unique sample-peptide enrichments are suggestive of a biological response when faced with a specific antigen and thus, revealing the profile of epitopes for each sample. The initial results presented provide evidence of SARS-CoV-2 specific epitopes along the Spike (S) and Nucleocapsid (N) proteins of the virus. In total, the authors identified 823 unique epitopes across the viral proteome. Severity of COVID-19 disease in an individual has been correlated with a variety of demographics and comorbidities [Yuki et al., 2020]. However, variance of infection outcome within these groups suggests also that an individual’s viral exposure history plays a role in combating infection. Shared epitopes across viruses is known as *cross-reactivity*. To understand which HCoV exposures may be cross-reactive with SARS-CoV-2 antibodies, the authors profiled pre-pandemic sample and found significant binding events to SARS-CoV-2 peptides. This result points out how exposure history plays a large role in infection outcome and may explain the large number of asymptomatic carriers of the disease.

Predicting SARS-CoV-2 infection history To understand the spread of a virus among a population, rapid testing for past infection is key. The binding profile of any individual’s antibodies to key proteins involved in infection give insight into their viral history and thus, provide an avenue to accurately detect previous infection of the SARS-CoV-2 virus. The authors presented a machine learning model that used z-score enrichment values as input and predicted the infection status of COVID-19. The model used the XGBoost algorithm which

results in a random forest of decision trees to create a binary classifier. The prediction model was trained on all of the sample’s serum samples using their respective infection status as either (1) COVID-19 positive or (2) pre-pandemic – as the target prediction. K-fold cross validation was used to reveal 99% Sensitivity and 98% specificity of the model. Shap plots were used to reveal the most significant peptides involved with the prediction. Interestingly, they found significant overlap with the epitopes specific to SARS-CoV-2 as being high in the decision tree model – supported the robustness of the model. These results provide evidence concerning the amount of information to be gained using antibody binding profiles from an individual to predict infection history.

Deep Mutational Scanning (DMS)

Understanding how underlying amino acid sequences relates to the function (*phenotype*) of proteins remains at large in many facets of biology. In the case immunology, we are interested in the process by which a pathogen leads to diseased state in an individual - known as *pathogenesis* [Araya and Fowler, 2011, Fowler and Fields, 2014, Weile and Roth, 2018]. We know, for instance, that more than half of human disease is caused by sequence mutations, or single nucleotide polymorphisms (SNPs) [Stenson et al., 2009]. Unfortunately, the lack of ability to map a sequence to the respective phenotype poses a large problem when developing therapeutics or predicting the pathogenic outcome of mutation in a pathogen. To address the difficulty, [Araya and Fowler, 2011] popularized a method which uses mutagenesis to explore how sequence variation changes the binding properties of protein relative to the wild type protein of interest – this method is known as Deep Mutational Scanning (DMS). This method generates a quantitative assay containing nearly every possible amino acid substitution along the protein of interest and then infers how each performs in vitro. If we consider binding as a key property of proteins, then the difference in binding affinity of mutations gives us a more full picture of how the underlying sequence relates to it’s phenotype. Presented in Adams et al. [2016], the authors show how this brute force approach to mutagenesis and subsequent measuring of binding affinity to another protein of interest allow us to explore the function of a protein at the sequence level.

In the context of viruses, each virus infects cells by binding to a protein on the surface of the host cell via a binding site of it’s own. It then follows that mutations to the binding sites on these viruses would regulate it’s affinity to bind, and thus, regulate the infectious properties of the virus. In Thyagarajan and Bloom [2014], the authors explore how the seasonal A/WSN/1933, H1N1 influenza virus is able to escape immunity through mutation. Using DMS, the authors were able to show how Influenza hemagglutinin – the flu’s integral protein for binding to host cells – has a high tolerance to mutation in regards to it’s binding affinity. In other words, mutations which may allow the virus to escape recognition and destruction by lymphocytes in the immune system, seem to consistently retain properties which allow for viral entry and subsequent infection of host cells. Below, we dive into the results from a study exploring the binding affinity of SARS-CoV-2 RBD mutants to the human ACE2 receptor.

Measuring binding affinity of SARS-CoV-2 RBD mutants to human ACE2 receptor The entry receptor on host cells for SARS-CoV-2 is the angiotensin converting enzyme 2 (ACE2). The Receptor Binding Domain (RBD) of the virus’ Spike (S) protein encodes for the protein allowing for viral entry as a result to binding to ACE2. Given this knowledge it would be very relevant to understand how sequence variants on the RBD impact the mutant’s binding affinity to the ACE2 receptor. Additionally, by assessing viral binding affinity of known variants in the human population today, we can provide evidence about the nature of selection currently acting on the virus. In Starr et al. [2020], the authors developed a yeast surface-display library of nearly all possible mutations along the Spike protein of SARS-CoV-2. They then measure the change in binding affinity to ACE2 when compared to the wild type sequence. Concretely, the authors performed PCR-induced mutagenesis to produce 3,804 of the 3,819 possible RBD amino-acid mutations of the protein coding sequence. Each variant produced was then linked to a unique 16bp barcode using long-read PacBio SMRT sequencing [Matreyek et al., 2018]. Next, the sequences were cloned into a library of yeast which then displayed the full tertiary structure of the respective proteins on the exterior of the fungus. Using techniques introduced in Adams et al. [2016] and Peterman and Levine [2016], the combination of fluorescent-activated cell sorting (FACS) and deep sequencing was used to measure the ACE2 expression levels – as well as their respective binding affinity to ACE2 – for each RBD variant in the libraries. By identifying the distribution of read counts among bins separated by level of RBD expression (measured using fluorescent intensity), Each variant’s mean fluorescent intensity (MFI) was identified. The binding affinities of each variant were presented as the log fold change when

compared to the wild type RBD MFI, $\Delta\log(MFI)$. Titration curves as described in Peterman and Levine [2016] were fit to each of the variants to infer dissociation constants, $K_{D,app}$ and were reported as $\Delta\log_{10}(K_{D,app})$. In short, this method allowed the authors to measure the binding affinity (fitness) of each nearly each of the possible 19 amino acid substitutions along the SARS-CoV-2 RBD, relative to the wild-type sequence.

Results of RBD variant binding affinity and viral evolution The measure of mutant binding affinity reveals many insights into mutational tolerance of the SARS-CoV-2 RBD. As expected, single amino acid substitutions are generally deleterious when compared with the wild type. This finding would comply with the well-established fact that amino acid substitutions generally impede the function and folding properties of most proteins as described in Soskine and Tawfik [2010]. However, the authors present the result that 46% of single amino-acid mutations along the RBD retain the ability to bind relatively well to ACE2 suggesting high mutational tolerance. With a large number of targeted epitopes in the RBD, this finding would suggest many potential mutational paths of escape from immunity in individuals – while retaining the ability to infect host cells. In contrast, many of the targeted epitopes have more mutational tolerance than those of the RBD in direct contact with ACE2, suggesting clever epitope targeting may limit the potential pathways of escape for SARS-CoV-2. To explore how existing mutations may be selected for currently, 31,570 sequences of isolated RBD sequences were observed from human samples to present 98 current RBD variants. Of all mutations found, 56 were observed in only a single sample presenting the low frequency of viral mutation and selection. The binding affinities of the observed mutations from human samples had significantly less deleterious effects when compared to random mutations, suggesting purifying selection among the viral population. Importantly, nearly all mutations present in human samples had nearly neutral change in binding affinity, which would seem to suggest there is no positive selection for more aggressive mutants in the population to date.

Conclusions and Discussion

In this literature review, we have explored the benefit of using quantitative assays (and their associated protocols) to gain insight into key properties of viral infections. We provide background and described difficulty of exploring protein function given underlying amino acid sequence. We explore studies focused on relevant proteins expressed by SARS-CoV-2 – the novel betacoronavirus responsible over a million deaths worldwide to date – as a motivating example for the use and advancement of these methods. We explain the history behind these methods and how each is an aggregate of several historical advancements in a large variety of molecular and computational techniques including but not limited to Next Generation Sequencing, Oligonucleotide Synthesis, and induced mutagenesis across a genetic sequence of interest. The urgency of combating disease sheds light on necessity of these quantitative assays to measure protein function and fitness in the context of viral immunology.

We summarized PhIP-Seq, a brute force approach to synthesizing the span of all possible proteins of interest across a proteome, and then subsequently measure their binding affinity to sampled antibodies. We reviewed Shrock et al. [2020] which explored, in detail, the landscape of SARS-CoV-2 epitopes revealed by antibodies from a large cohort of individual serum antibodies. This study provided insight into the specific epitopes unique to SARS-CoV-2 generated antibodies as well cross-reactive epitopes with related coronaviruses. The data generated from this protocol gave enough statistical power to outperform state-of-the-art SARS-CoV-2 infection history testing.

This review explained the basic methods underlying DMS and its benefits when exploring mutational impact on protein binding affinity to another protein of interest. While we explored this method in the context of viral immunology, it should be noted that this technique has broad implications and potential for exploring genotype to phenotype maps at the level of proteins more universally. By exploring the key results from Starr et al. [2020], we described evidence about the current forces of evolution acting on the SARS-CoV-2 virus in humans. Additionally, the evidence provided suggested that the RBD of SARS-CoV-2 had high mutational tolerance with respect to its ability to retain viral entry functionality. Fortunately, the data provided may help design broadly neutralizing antibodies limiting escape, and thus allowing us to confer long-term immunity against the virus.

For brevity, this review avoids the complex analysis and computational techniques necessary for exploring the large resulting datasets. Instead, we focus on the background, explanation, and relevant results from novel studies. Unfortunately, this overlooked an aspect of these protocols which currently acts as a bottleneck in exploring the resulting data. Indeed, both PhIP-Seq and DMS give us datasets which are relatively large and

noisy – highlighting accurate modeling a necessity in order to avoid false positive results from inherent bias introduced by various steps of each respective protocol.

In conclusion, this literature review hopes to explain and motivate the power of quantitative assays when exploring protein function in viral immunology. It does not critique many aspects of each method which act as shortcomings in their ability to clearly provide results. PhIP-Seq provides a platform that gives a profile of antibody binding affinity for each sample which is presented to the library. When the samples are combined, patterns can be observed to provide insight into the biological mechanisms of the immune system in response to a given virus. Researchers can leverage this information to develop the most effective vaccines and therapeutics to target the pathogen and prevent its proliferation in individuals. DMS has laid the foundation for exploring protein function as a whole simply observing it's change in function when compared to all possible 19 amino acid substitutions. The information about protein variants gives us crucial information about the current forces of evolution of a virus in real time during a pandemic such as the spread of COVID-19. Together, these results provide motivation for using quantitative assays in viral immunology.

References

- Rhys M Adams, Thierry Mora, Aleksandra M Walczak, and Justin B Kinney. Measuring the sequence-affinity landscape of antibodies with massively parallel titration curves. *eLife*, 5:e23156, dec 2016. ISSN 2050-084X. doi: 10.7554/eLife.23156. URL <https://doi.org/10.7554/eLife.23156>.
- Carlos L. Araya and Douglas M. Fowler. Deep mutational scanning: assessing protein function on a massive scale. *Trends in Biotechnology*, 29(9):435 – 442, 2011. ISSN 0167-7799. doi: <https://doi.org/10.1016/j.tibtech.2011.04.003>. URL <http://www.sciencedirect.com/science/article/pii/S0167779911000692>.
- J Felsenstein. Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.*, 17(6): 368–376, 1981. ISSN 0022-2844. URL <http://www.ncbi.nlm.nih.gov/pubmed/7288891>.
- Douglas M. Fowler and Stanley Fields. Deep mutational scanning: a new style of protein science. *Nature Methods*, 11(8):801–807, Aug 2014. ISSN 1548-7105. doi: 10.1038/nmeth.3027. URL <https://doi.org/10.1038/nmeth.3027>.
- Meghan E. Garrett, Jared Galloway, Helen Y. Chu, Hannah L. Itell, Caitlin I. Stoddard, Caitlin R. Wolf, Jennifer K. Logue, Dylan McDonald, Frederick A. Matsen, and Julie Overbaugh. High resolution profiling of pathways of escape for sars-cov-2 spike-binding antibodies. *bioRxiv*, 2020. doi: 10.1101/2020.11.16.385278. URL <https://www.biorxiv.org/content/early/2020/11/16/2020.11.16.385278>.
- David Jung and Frederick W. Alt. Unraveling v(d)j recombination: Insights into gene regulation. *Cell*, 116(2):299–311, Jan 2004. ISSN 0092-8674. doi: 10.1016/S0092-8674(04)00039-X. URL [https://doi.org/10.1016/S0092-8674\(04\)00039-X](https://doi.org/10.1016/S0092-8674(04)00039-X).
- H. Benjamin Larman, Zhenming Zhao, Uri Laserson, Mamie Z. Li, Alberto Ciccia, M. Angelica Martinez Gakidis, George M. Church, Santosh Kesari, Emily M. Leproust, Nicole L. Solimini, and Stephen J. Elledge. Autoantigen discovery with a synthetic human peptidome. *Nature biotechnology*, 29(6):535–541, May 2011. ISSN 1546-1696. doi: 10.1038/nbt.1856. URL <https://pubmed.ncbi.nlm.nih.gov/21602805>. 21602805[pmid].
- Kenneth A. Matreyek, Lea M. Starita, Jason J. Stephany, Beth Martin, Melissa A. Chiasson, Vanessa E. Gray, Martin Kircher, Arineh Khechaduri, Jennifer N. Dines, Ronald J. Hause, Smita Bhatia, William E. Evans, Mary V. Relling, Wenjian Yang, Jay Shendure, and Douglas M. Fowler. Multiplex assessment of protein variant abundance by massively parallel sequencing. *Nature genetics*, 50(6):874–882, Jun 2018. ISSN 1546-1718. doi: 10.1038/s41588-018-0122-z. URL <https://pubmed.ncbi.nlm.nih.gov/29785012>. 29785012[pmid].
- Divya Mohan, Daniel L. Wansley, Brandon M. Sie, Muhammad S. Noon, Alan N. Baer, Uri Laserson, and H. Benjamin Larman. Phip-seq characterization of serum antibodies using oligonucleotide-encoded peptidomes. *Nature Protocols*, 13(9):1958–1978, Sep 2018. ISSN 1750-2799. doi: 10.1038/s41596-018-0025-6. URL <https://doi.org/10.1038/s41596-018-0025-6>.
- Ahmad Abu Turab Naqvi, Kisa Fatima, Taj Mohammad, Urooj Fatima, Indrakant K. Singh, Archana Singh, Shaikh Muhammad Atif, Gururao Hariprasad, Gulam Mustafa Hasan, and Md Imtaiyaz Hassan. Insights

- into sars-cov-2 genome, structure, evolution, pathogenesis and therapies: Structural genomics approach. *Biochimica et biophysica acta. Molecular basis of disease*, 1866(10):165878–165878, Oct 2020. ISSN 1879-260X. doi: 10.1016/j.bbadis.2020.165878. URL <https://pubmed.ncbi.nlm.nih.gov/32544429>. 32544429[pmid].
- Neil Peterman and Erel Levine. Sort-seq under the hood: implications of design choices on large-scale characterization of sequence-function relations. *BMC Genomics*, 17(1):206, Mar 2016. ISSN 1471-2164. doi: 10.1186/s12864-016-2533-5. URL <https://doi.org/10.1186/s12864-016-2533-5>.
- Ellen Shrock, Eric Fujimura, Tomasz Kula, Richard T. Timms, I-Hsiu Lee, Yumei Leng, Matthew L. Robinson, Brandon M. Sie, Mamie Z. Li, Yuezhou Chen, Jennifer Logue, Adam Zuiani, Denise McCulloch, Felipe J. N. Lelis, Stephanie Henson, Daniel R. Monaco, Meghan Travers, Shaghayegh Habibi, William A. Clarke, Patrizio Caturegli, Oliver Laeyendecker, Alicja Piechocka-Trocha, Jon Li, Ashok Khatrri, Helen Y. Chu, , Alexandra-Chloé Villani, Kyle Kays, Marcia B. Goldberg, Nir Hacohen, Michael R. Filbin, Xu G. Yu, Bruce D. Walker, Duane R. Wesemann, H. Benjamin Larman, James A. Lederer, and Stephen J. Elledge. Viral epitope profiling of covid-19 patients reveals cross-reactivity and correlates of severity. *Science*, 2020. ISSN 0036-8075. doi: 10.1126/science.abd4250. URL <https://science.sciencemag.org/content/early/2020/09/28/science.abd4250>.
- Misha Soskine and Dan Tawfik. Mutational effects and the evolution of new protein functions. *Nature reviews. Genetics*, 11:572–82, 08 2010. doi: 10.1038/nrg2808.
- Tyler N. Starr, Allison J. Greaney, Sarah K. Hilton, Daniel Ellis, Katharine H.D. Crawford, Adam S. Dingens, Mary Jane Navarro, John E. Bowen, M. Alejandra Tortorici, Alexandra C. Walls, Neil P. King, David Vesler, and Jesse D. Bloom. Deep mutational scanning of sars-cov-2 receptor binding domain reveals constraints on folding and ace2 binding. *Cell*, 182(5):1295 – 1310.e20, 2020. ISSN 0092-8674. doi: <https://doi.org/10.1016/j.cell.2020.08.012>. URL <http://www.sciencedirect.com/science/article/pii/S0092867420310035>.
- Peter D. Stenson, Matthew Mort, Edward V. Ball, Katy Howells, Andrew D. Phillips, Nick ST Thomas, and David N. Cooper. The human gene mutation database: 2008 update. *Genome Medicine*, 1(1):13, Jan 2009. ISSN 1756-994X. doi: 10.1186/gm13. URL <https://doi.org/10.1186/gm13>.
- Caitlin I. Stoddard, Jared Galloway, Helen Y. Chu, Mackenzie M. Shipley, Hannah L. Itell, Caitlin R. Wolf, Jennifer K. Logue, Ariana Magedson, Kevin Sung, Meghan Garrett, Katharine H.D. Crawford, Uri Laserson, Frederick A. Matsen, and Julie Overbaugh. Epitope profiling reveals binding signatures of sars-cov-2 immune response and cross-reactivity with endemic hcovs. *bioRxiv*, 2020. doi: 10.1101/2020.10.29.360800. URL <https://www.biorxiv.org/content/early/2020/10/29/2020.10.29.360800>.
- Bargavi Thyagarajan and Jesse D Bloom. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. *eLife*, 3:e03300, jul 2014. ISSN 2050-084X. doi: 10.7554/eLife.03300. URL <https://doi.org/10.7554/eLife.03300>.
- S. Susanna Twiddy, Edward C. Holmes, and Andrew Rambaut. Inferring the Rate and Time-Scale of Dengue Virus Evolution. *Molecular Biology and Evolution*, 20(1):122–129, 01 2003. ISSN 0737-4038. doi: 10.1093/molbev/msg010. URL <https://doi.org/10.1093/molbev/msg010>.
- Jochen Weile and Frederick P. Roth. Multiplexed assays of variant effects contribute to a growing genotype–phenotype atlas. *Human Genetics*, 137(9):665–678, Sep 2018. ISSN 1432-1203. doi: 10.1007/s00439-018-1916-x. URL <https://doi.org/10.1007/s00439-018-1916-x>.
- Fan Wu, Su Zhao, Bin Yu, Yan-Mei Chen, Wen Wang, Zhi-Gang Song, Yi Hu, Zhao-Wu Tao, Jun-Hua Tian, Yuan-Yuan Pei, Ming-Li Yuan, Yu-Ling Zhang, Fa-Hui Dai, Yi Liu, Qi-Min Wang, Jiao-Jiao Zheng, Lin Xu, Edward C. Holmes, and Yong-Zhen Zhang. A new coronavirus associated with human respiratory disease in china. *Nature*, 579(7798):265–269, Mar 2020. ISSN 1476-4687. doi: 10.1038/s41586-020-2008-3. URL <https://doi.org/10.1038/s41586-020-2008-3>.
- George J. Xu, Tomasz Kula, Qikai Xu, Mamie Z. Li, Suzanne D. Vernon, Thumbi Ndung’u, Kiat Ruxrungtham, Jorge Sanchez, Christian Brander, Raymond T. Chung, Kevin C. O’Connor, Bruce Walker, H. Benjamin Larman, and Stephen J. Elledge. Viral immunology. comprehensive serological profiling of human populations using a synthetic human virome. *Science (New York, N.Y.)*, 348(6239):aaa0698–aaa0698, Jun 2015. ISSN 1095-9203. doi: 10.1126/science.aaa0698. URL <https://pubmed.ncbi.nlm.nih.gov/26045439>. 26045439[pmid].

Supplementary Figures

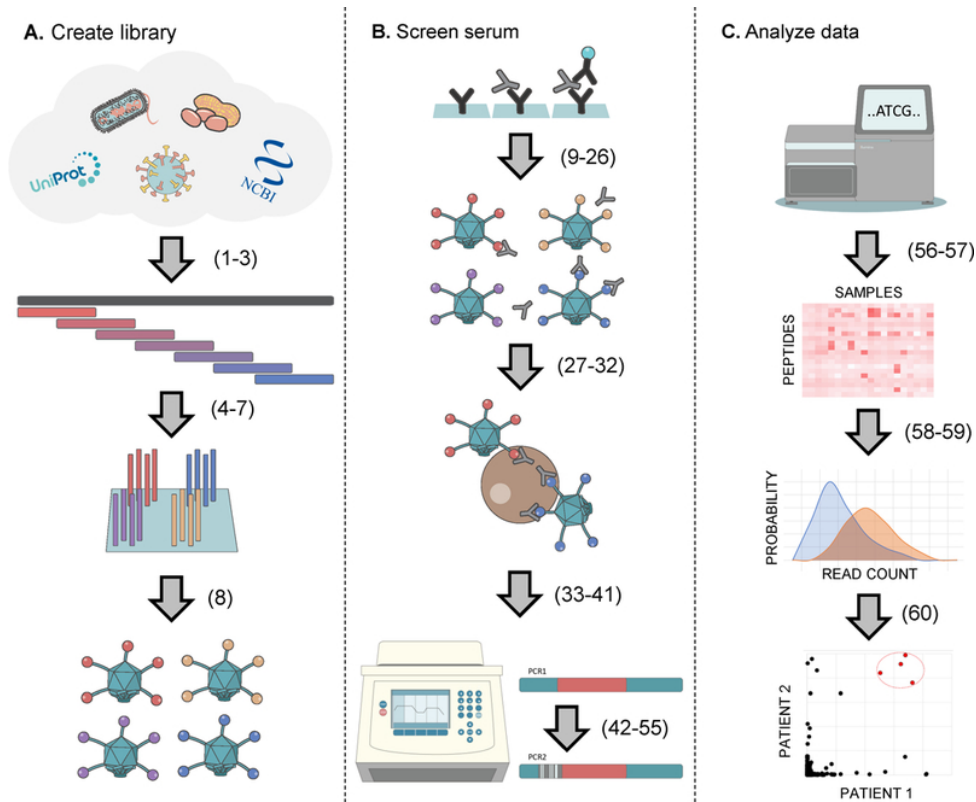


Figure 1: lsjfln

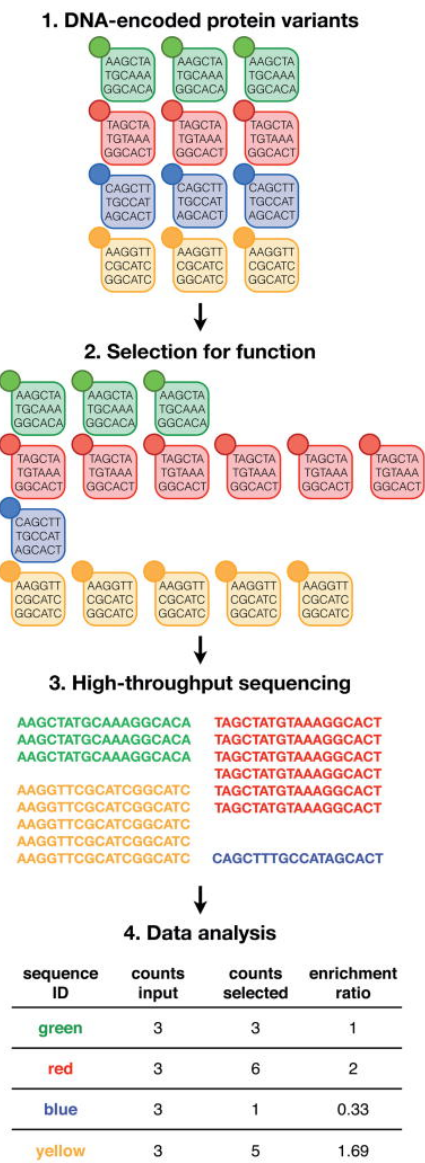


Figure 2: lsjfln

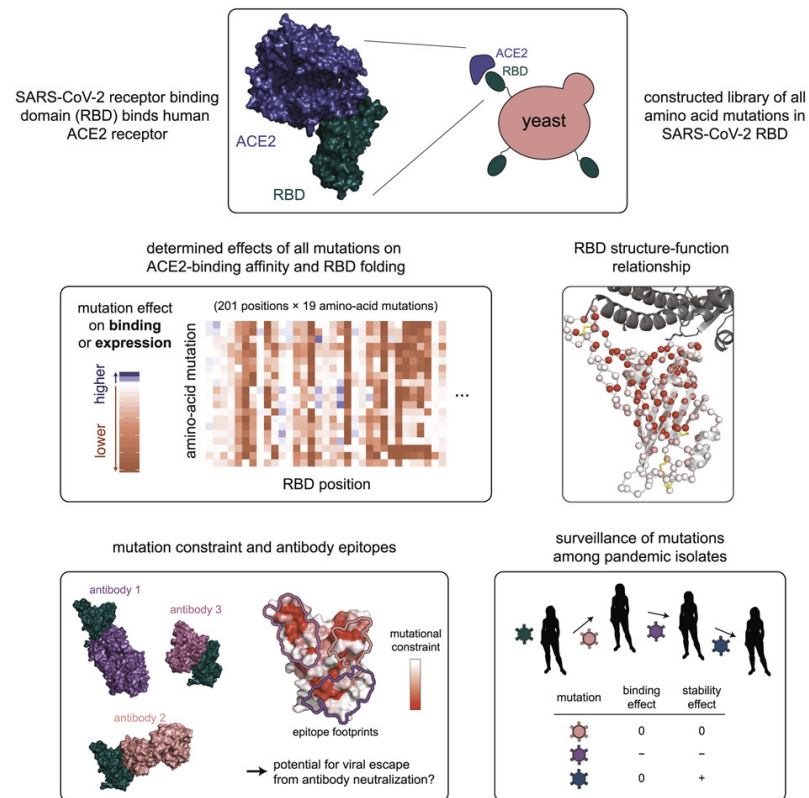


Figure 3: lsjfln

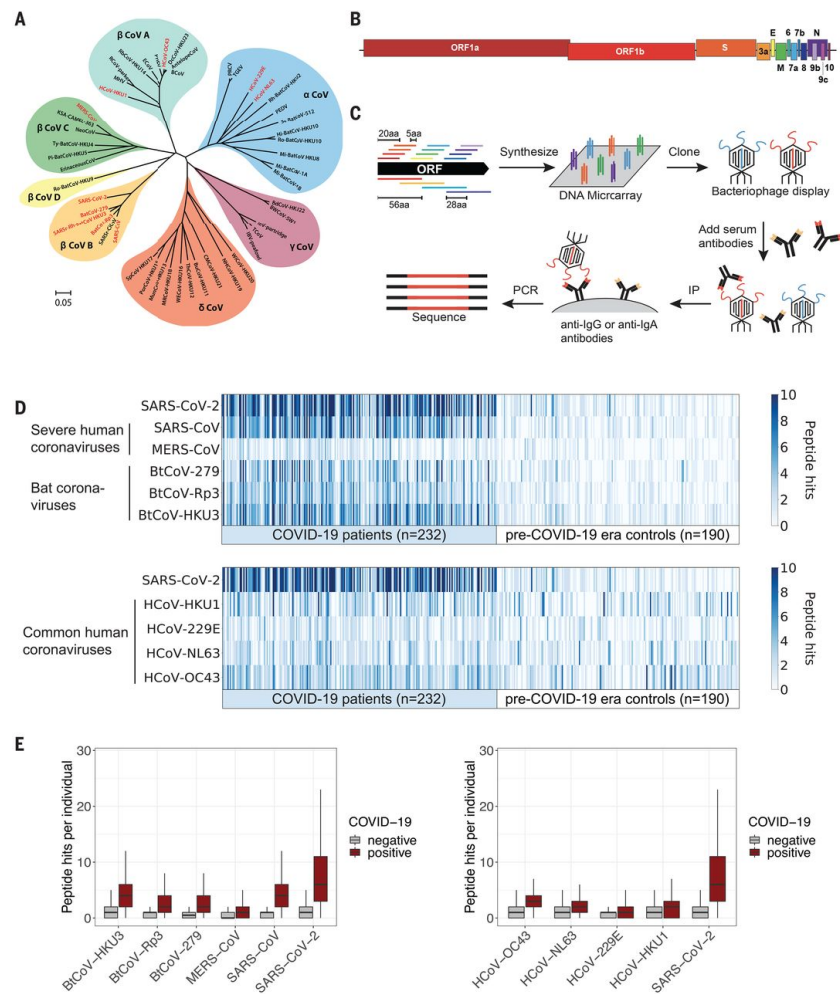
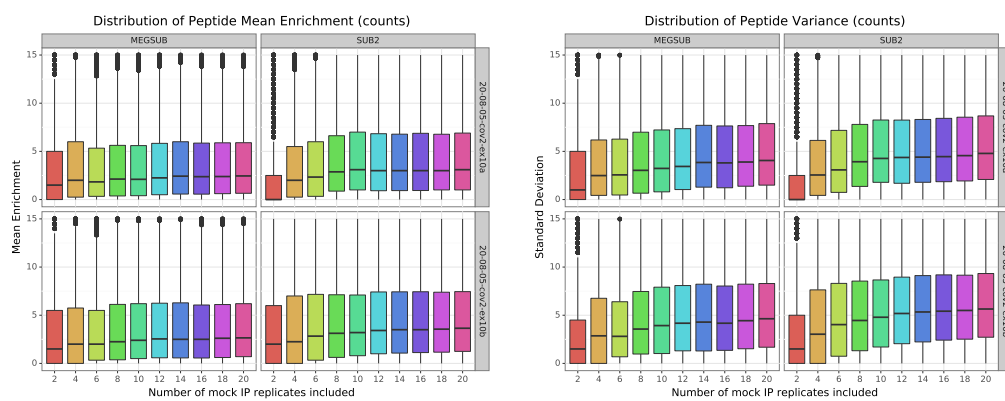
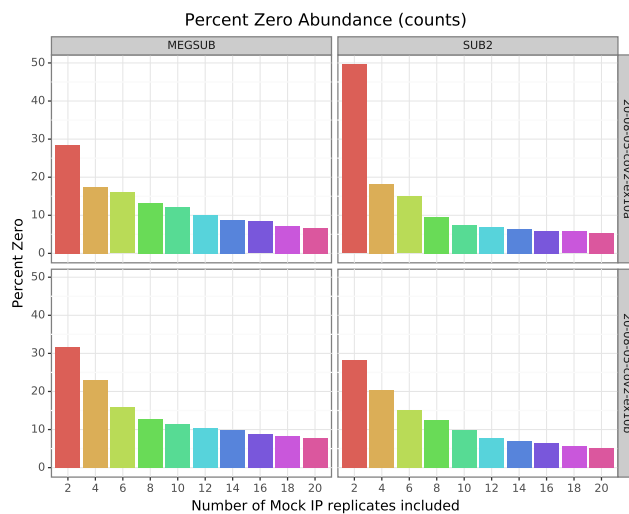


Figure 4: lsjfln



(a) first

(b) second



(e) fifth

Figure 5: caption