Immunology and Inflammation
Computational and Systems Biology

PAbFold: Linear Antibody Epitope Prediction using AlphaFold2

Reviewed Preprint
v2 • January 23, 2025
Revised by authors

Reviewed Preprint
v1 • June 11, 2024

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eLife Assessment

In this manuscript, the authors describe a new AlphaFold2 pipeline called PabFold that can represent a **useful** tool for identifying linear antibody epitopes (B-cell epitopes) for different antigens. This information can be used in the selection of different reagents in competitive ELISA assays which can save time and reduce costs. Because several questions about the work remain, the study is currently **incomplete**.

https://doi.org/10.7554/eLife.98369.2.sa3

Abstract

Defining the binding epitopes of antibodies is essential for understanding how they bind to their antigens and perform their molecular functions. However, while determining linear epitopes of monoclonal antibodies can be accomplished utilizing well-established empirical procedures, these approaches are generally labor- and time-intensive and costly. To take advantage of the recent advances in protein structure prediction algorithms available to the scientific community, we developed a calculation pipeline based on the localColabFold implementation of AlphaFold2 that can predict linear antibody epitopes by predicting the structure of the complex between antibody heavy and light chains and target peptide sequences derived from antigens. We found that this AlphaFold2 pipeline, which we call PAbFold, was able to accurately flag known epitope sequences for several well-known antibody targets (HA / Myc) when the target sequence was broken into small overlapping linear peptides and antibody complementarity determining regions (CDRs) were grafted onto several different antibody framework regions in the single-chain antibody fragment (scFv) format. To determine if this pipeline was able to identify the epitope of a novel antibody with no structural information publicly available, we determined the epitope of a novel anti-SARS-CoV-2 nucleocapsid targeted antibody using our method and then experimentally validated our computational results using peptide competition ELISA assays. These results indicate that the AlphaFold2-based PAbFold pipeline we developed is capable of accurately identifying linear antibody epitopes in a short time using just antibody and target protein sequences. This emergent capability of the method is sensitive to methodological details such as peptide

length, AlphaFold2 neural network versions, and multiple-sequence alignment database. PAbFold is available at https://github.com/jbderoo/PAbFold.

Introduction

Understanding where and how an antibody binds to its target protein is important for understanding how the antibody performs its function, whether that function is neutralizing a pathogen during an immune response, binding an epitope in immunoassays, or labeling a target molecule in a live-cell imaging experiment. However, determining the binding epitope of an antibody can be a time and labor-intensive endeavor with significant expense. Traditionally, antibody epitopes on target proteins have been identified by performing deletion analysis on the target protein to determine if the antibody loses reactivity for the deletion mutants in various immunoassays, which provides the general region of the target protein the antibody binds to. With the advent of widely available chemical peptide synthesis, sequence-specific synthetic peptides can be used for competitive immunoassays (such as enzyme-linked immunosorbent assays (ELISA)) to establish sequences that can effectively compete with the antigen for antibody binding. Peptide mapping experiments are a powerful method for determining the fine sequence of linear antibody epitopes, but these experiments can be relatively expensive and the time between experimental design and data acquisition can be weeks to months due to the need to design and chemically synthesize peptides. Once a peptide has been identified that binds with high affinity and specificity to an antibody antigen binding fragment (Fab), crystal structures can be determined that demonstrate intermolecular interactions between the peptide and antibody. These can then provide a molecular-level explanation for an antibody's binding mode. Finally, with the advent of rapid single B-cell sequencing technologies to analyze humoral immune responses towards vaccination or infection, determining where specific antibody clones bind on an antigen becomes even more challenging due to the need to isolate or synthesize specific antibody genes, produce antibodies, and then perform deletion or epitope mapping experiments described above to fully understand how and where antibodies bind. These challenges make determining antibody epitopes expensive and time-consuming and limit the number of antibodies that are characterized in detail.

Antibodies that bind to linear epitopes represent an important subset to molecular biology, as they can be added to recombinant proteins for use in various types of immunoassays. By definition, a linear epitope is a binding site on an antigen that is recognized by the primary structure or contiguous linear sequence of amino acids. A number of linear epitope specific antibodies have been developed for use in various immunoassays (ELISA, western blot, immunofluorescence, etc.). The development of computational methods for linear epitope determination could increase the number and quality of new linear epitopes available to the field. Most epitope prediction tools (such as BepiPred (1), ElliPro (2), and ABCpred (3)) are generally designed to predict regions of an antigen that could be recognized by any antibody rather than a specific antibody. These programs also provide no insight into the structural match of the epitope and antibody, potentially making decisions without key structural information that otherwise may be relevant. The challenge in predicting epitopes for a *specific* antibody lies in the complexity of protein-protein interaction dynamics, which includes conformational changes, binding affinities, and thermodynamic stability. Structure based approaches including HADDOCK (4, 5) and ZDOCK (4, 6) can be used to dock peptides into antibody structures, but these require known peptides for binding. Significant progress has been made to address this problem via deep learning: some of the new and exciting tools are GearBind (7), PALM and A2binder (8), and DSMBind (9). We point the reader to this review for an excellent overview of some of the tools that have existed for some time, along with a comparison of these tools (10).

Determining antibody-epitope interactions is, at its most basic level, a structural biology problem. Determining what molecular interactions are present between an antibody and its antigen can define the epitope, determine what portions of the epitope and CDR sequences are responsible for molecular interactions, and provide clues to antibody specificity and affinity. With the advent of highly accurate structural predictions, including the AlphaFold2 (AF2) neural networks (11, 12), the ability to accurately predict protein structures, and potential protein-protein interactions, has dramatically increased. AlphaFold2 was trained on existing protein structures and can effectively model new protein structures.

Numerous antibodies, antibody Fab regions, and other related constructs with bound target peptides or proteins have been crystalized and deposited into the Protein Data Bank (PDB) (for example (13–16)). These PDB entries represent a valuable training set that may increase the likelihood that AlphaFold2 can successfully predict the structure for antibody-epitope complexes (12, 17–19). The authors of AlphaFold2 multimer (12) comment on the difficulty of predicting antibody-epitope complexes, and results for this are indeed mixed at best (17–19). One way in which this current report is distinct is our focus on linear epitopes. We hypothesize that the lack of strong competing structure within the short peptide may boost AF2 prediction of scFv-epitope binding predictions relative to conformational epitopes. This problem has precedent, as AlphaFold2 has previously been used to study the interactions between proteins and peptides (17, 18). AlphaFold2's ability to correctly dock independent protein chains can be repurposed to predict how strongly two proteins interact together and extends to predicting the interaction between an antibody and short flexible peptides (linear epitopes) drawn from a larger protein antigen.

To maximize compute efficiency, it is helpful to minimize the size of the system subject to structure prediction. The computational expense of AlphaFold2 scales with the square of the length of the concatenated sequences involved. Fortunately, with respect to epitope specificity, antibody constant domains are less critical than the CDR loops and the remainder of the variable domain framework regions. Antigen binding by antibodies is primarily dictated by the antigen binding fragment (Fab) containing the variable light (VL) and variable heavy (VH) fragments. Conversion of full antibody sequences into single chain variable fragments (scFv) can significantly reduce structure prediction complexity and compute time. A wildtype scFv sequence can easily be generated directly from translated antibody heavy and light chain DNA sequences. Briefly, the sequences are first divided into framework and complementarity determining regions (CDRs) using Kabat (20) or IMGT (21) nomenclature. A flexible linker sequence (GGGGSGGGGSGGGGS, 15 a.a.) is then added between the new C-terminus of the truncated light chain and the original N-terminus of the shortened heavy chain to generate a single protein sequence that incorporates both antigen-binding chains. The resulting fusion protein often functions in a similar fashion to the original antibody. Another well-known protein engineering strategy for antibodies is "loop grafting", where the CDR loops from one antibody are grafted onto a different framework region. We have recently used this approach to develop scFvs with improved *in vivo* performance (22). The structures of the novel scFv chimeras can be rapidly and confidently predicted by AlphaFold2 due to their small size and the extensive immunoglobin representation within sequence databases and the PDB. Excluding the time needed to obtain a multiple sequence alignment (MSA), predicting the structure for a single scFv in complex with a 10-a.a. peptide requires only 1.5 minutes on an NVIDIA A5000 graphics processing unit (GPU). This modest compute time allows a GPU-laden server or workstation to handle large-scale structure prediction of hundreds of related systems. As for the MSA input, a high quality MSA can quickly be obtained via ColabFold (23), which relies on the MMseqs2 MSA server. In our workflow, we repeatedly predict the structure for a fixed single scFv sequence in complex with varying peptide partners. In this case, we do not expect the peptide portion of the MSA to be useful. Therefore, to avoid sending hundreds of nearly identical MSA requests to MMseqs2 MSA server, and to avoid

varying information in the MSA, we slightly modified the LocalColabFold code to include the option to cache the MSA (install available on the GitHub). We generate one cached MSA per epitope scan, where each residue in the query peptide is a glycine.

Several recent papers have attempted to use AlphaFold2 to identify antibody epitopes (24–26), but have primarily focused on computational identification and have not verified their results using new antibodies that are not within the PDB training set. While there are many other structure prediction models other than AlphaFold2 (27, 28), including some specifically dedicated to predicting antibodies or antibody-like structures (29–32), we chose AlphaFold2 to directly test its ability to correctly identify and place epitopes into an antibody binding cleft. We selected AlphaFold2 due to its widespread use throughout the literature, as well as its ease of installation and modification via the LocalColabFold implementation (23). Another reason for selecting AF2 is to attempt to quantify its abilities the compare simple linear epitopes, since the team behind AF-multimer reported that conformational antibody complexes were difficult to predict accurately (12). In this project we test a method we call PAbFold, a LocalColabFold-based pipeline to identify epitopes for several well-known linear-epitope antibodies from sequence information only. There was a strong correlation between AlphaFold2's confidence in the peptide structure (pLDDT) (33) and the experimentally verified epitope binding sequence. Additionally, we found that AlphaFold2 very accurately predicted the linear epitope of a novel SARS-CoV-2 nucleocapsid-specific antibody (mBG17) with minimal prior epitope information. The molecular interactions predicted by AlphaFold2 were experimentally validated using peptide mapping ELISA experiments. Overall, this work demonstrates that AlphaFold2 has compelling promise for linear antibody epitope discovery from sequence information alone. We also have observed that this emergent linear epitope prediction ability is sensitive to the peptide length and that the performance was optimal when using AlphaFold2-multimer version 2 and older MSAs generated by MMSEQS version 2202 server, rather than the more recent AlphaFold2-multimer version 3 models and MMSEQS version 2302 server.

Materials and methods

Software

All structure predictions were completed on a single AMD EPYC 7443 server with two NVIDIA RTX A5000 GPU cards. PAbFold code was written in Python 3.7 and Bash. The only extra Python dependencies are NumPy and Matplotlib. AlphaFold2 calculations were run using an installation of LocalColabFold (23). Briefly, PAbFold contains 3 stages. In the first stage, a python script 'A_PeptideMapping_prep_submission_files.py' writes FASTA input files for ColabFold. Each FASTA file contains the entire sequence of the subject scFv, a colon ":", and then the candidate linear epitope which represents a small section of the target antigen protein that changes dependent upon both the epitope length (default 10 a.a.) and a sliding window (default 1 a.a.).

After completion of the ColabFold jobs, two different analysis methods are presented in this paper, and both are accessible via the 'B_PeptideMapping_plddt_perres_analysis.py' python script. The first is the 'Simple Max' method, which assesses each peptide window with only the output model that is top ranked by ColabFold (on the basis of ipTM). The AlphaFold2 confidence pLDDT (33) is recorded for each residue within the peptide. Other than the N- and C-terminal residues, each residue is observed within multiple windows. We proceed to calculate (and plot) the maximum pLDDT observed for each residue across the set of sliding window peptides that contain that residue. Thus, in the 'Simple Max' method each residue is considered independently. To obtain aggregate scores for each peptide window, we sum the maximum pLDDT associated with each member residue. This method is sensitive in that any isolated high-confidence residue placements in the top ranked AlphaFold2 peptide prediction can increase the score, but a high aggregate peptide score could arise from multiple, mutually inconsistent peptide binding poses. Our second,

complementary analysis method instead focuses on recognizing full peptide poses of elevated AlphaFold2 confidence. We refer to the second method as the 'Consensus method' because it begins by averaging the per-residue pLDDT across the five AlphaFold2 models. We then compute the average pLDDT for each peptide. For visual inspection, scripts output a heat map for the average per-residue pLDDT and a bar-chart that for the subsequent per-peptide average pLDDT. In this case, we simply rank top peptides based on the per-peptide average pLDDT. Scripts are available at *https://github.com/jbderoo/PAbFold*.

Antibody sequences

Sequences and references for antibodies, scFvs, and antigens can be found in **Supplemental Table 1A**. To create an scFv, the complementarity determining regions or loops of an antibody are identified via the Kabat numbering scheme. The loops are then spliced onto the scFv backbones of the 15F11 and 2E2 as previously described by our group (22). The scFv sequences are aligned with their CDR loops and flexible linkers highlighted in **Supplemental Table 1B**.

Monoclonal Antibody Production

Anti-SARS-CoV-2 nucleocapsid protein (NP) monoclonal mouse antibody mBG17 was previously developed and characterized (34). Briefly, two BALB/c mice immunized with recombinant NP were sacrificed and primary splenocytes isolated. Splenocytes were fused with Sp2/0 Ag14 myeloma cells and individual hybridoma clones were isolated after eleven days. Hybridoma clones were tested for antibody production against NP via enzyme-linked immunosorbent assay (ELISA) and western blot. Clones were further tested for isotype and cross-reactivity, and VH and VL sequences were determined. The hybridoma clone mBG17 was identified as a SARS-CoV-2 nucleocapsid-specific antibody targeting linear epitope via ELISA and western blot (34). Generation of recombinant mBG17 and production of recombinant antibody in 293F cells was previously described (34). The approximate epitope region for mBG17 was determined via western blot with modified recombinant NP proteins containing 40 to 50 amino acid deletions. The epitope location was determined to reside between SARS-CoV-2 nucleocapsid residues a.a. 381-419 based on loss of western blot signal with the a.a. 381-419 deletion (34).

Peptide Competition ELISA

The anti-SARS-CoV-2 nucleocapsid protein mBG17 antibody epitope was experimentally identified using competition enzyme-linked immunosorbent assay (ELISA). Using the previously determined 39 nucleocapsid protein amino acid range for the mBG17 epitope as a starting point, seven overlapping peptides were synthesized (Thermo Scientific) spanning the 39 amino acid region with overlaps of 5 amino. These peptides were termed Fragment 1 through 7 (**Table 1**). A 96-well ELISA plate was coated with 0.1ug/ml of recombinant SARS-CoV-2 NP (34) overnight at 4°C. The plate was blocked with 4% (w/v) dry non-fat milk in 1X PBS with 0.1% (v/v) Tween-20 for 1 h shaking at room temperature. While blocking, inhibited recombinant mBG17 antibody samples were produced by incubating 40 µL of antibody with 40 µg (approximately 30 nMol) of a single peptide fragment for one hour at room temperature. Following this, peptide-incubated mBG17 was applied to the blocked nucleocapsid protein coated plate in triplicate and allowed to incubate for 1 h at room temperature while shaking. The plate was rinsed with 0.1% (v/v) Tween-20 in 1X PBS and washed three more times for 5 minutes shaking at room temperature. The plate was then incubated with HRP-conjugated goat anti-mouse polyclonal antibody solution diluted at 1:20,000 in 1X PBS for 1 h shaking at room temperature. After another rinse and three more washes the plate was developed with 1-Step™ Ultra TMB-ELISA Solution (ThermoFisher) before stopping the reaction with an equal volume of 2M H2SO4. Solution absorbance at 450 nm was measured using a PerkinElmer Victor X5 multilabel plate reader. Absorbances were averaged within fragment-inhibited sample groups and corrected with the average value of the negative control. These absorbances were then normalized against the absorbance from the group with the highest value before multiplying by 100 to obtain percentage of potential signal.

Peptide Name	Peptide Sequence
Nucleocapsid a. a. 381-390 (Frag 1)	ALPQRQKKQQ
Nucleocapsid a. a. 386-395 (Frag 2)	QKKQQTVTLL
Nucleocapsid a. a. 391-400 (Frag 3)	TVTLLPAADL
Nucleocapsid a. a. 396-405 (Frag 4)	PAADLDDFSK
Nucleocapsid a. a. 401-410 (Frag 5)	DDFSKQLQQS
Nucleocapsid a. a. 406-415 (Frag 6)	QLQQSMSSAD
Nucleocapsid a. a. 411-419 (Frag 7)	MSSADSTQA
Nucleocapsid D401A	ADFSKQLQQS
Nucleocapsid D402A	DAFSKQLQQS
Nucleocapsid F403A	DDASKQLQQS
Nucleocapsid S404A	DDFAKQLQQS
Nucleocapsid K405A	DDFSAQLQQS
Nucleocapsid Q406A	DDFSKALQQS
Nucleocapsid L407A	DDFSKQAQQS
Nucleocapsid Q408A	DDFSKQLAQS
Nucleocapsid Q409A	DDFSKQLQAS
Nucleocapsid S410A	DDFSKQLQQA

Table 1

The effect of single alanine substitutions on fragment 5 (DDFSKQLQQS) peptide binding was determined by competition ELISA using a series of ten alanine-substituted peptides (**Table 1**) at a range of concentrations to determine relative competition activity. A modified version of the previously described inhibition ELISA was performed using the unmodified Fragment 5 peptide and the ten alanine-substituted peptides. During the mBG17 inhibition step, the mBG17 antibody solution was incubated with a 4-fold serial dilution of peptides beginning at 40 µg and continuing to ~2.5 ng before being applied to the NP coated plates in triplicate. The remainder of the competition ELISA was carried out as described above.

Assessment of AlphaFold2 generated scFv structures

We first verified that AlphaFold2 could generate scFv structures that have similar structures to their parent monoclonal antibodies. We chose the 9E10 clone of the anti-Myc antibody as an initial test system, as the scFv sequence is available (35) and has a well-known linear epitope (EQKLISEEDL)(36). We predicted the wild-type Myc scFv structure and aligned this model to the corresponding Fab crystal structure (PDB entry 2orb) via the align command in PyMOL (**Supplemental Figure 1A**). The AlphaFold2 predicted scFv was very similar (RMSD value of 0.42Å) to the anti-Myc Fab structure, suggesting that the predicted scFv structure was a suitable starting point for epitope prediction. We also examined the structures of the Myc CDRs loop grafted onto the 15F11 (37) and 2E2 (22) frameworks, as we have previously observed that loop grafting onto these frameworks can enhance protein folding and solubility (22). The loop-grafted Myc-2E2 and Myc-15F11 and structures were also similar to the Myc Fab structure (PDB 2ORB) (36) with similar RMSD values of 0.45Å (**Supplemental Figure 1B**), indicating that they are also reasonable starting points for epitope prediction.

Results

Development of Python-based scripts for automated scFv:peptide structure prediction

We developed a series of Python scripts that automate the process of epitope prediction and analysis with AF2. A_Peptide_Mapping_prep_submission_files.py accepts a linear scFv sequence and a linear full-length antigen sequence, and processes the antigen sequence into a series of short peptides with custom peptide length and sliding window sizes (default parameters are 10 amino acid peptides with a 1 amino acid sliding window). It then adds lines for each scFv:peptide pair to a FASTA file. Structures are then predicted via LocalColabFold for each scFv:peptide pair with AlphaFold2 in parallel on two NVIDIA RTX A5000 GPUs. The python script B_PeptideMapping_plddt_perres_analysis.py parses the AlphaFold2 output structures to extract per-residue pLDDT for the peptide residues in each scFv:peptide pair. Conf_plot_and_top10.py will plot the maximum pLDDT (across all host peptides) scores as a function of amino acid position within the antigen sequence and ranks predicted peptides based on ΣpLDDT scores for the 'Simple max' method. To use the 'Consensus' method, include the –all-models flag when running B_PeptideMapping_plddt_perres_analysis.py. We also supply a python script that replicates how we present the data called all_model_analysis.py for use.

An overview of the method is shown in **Figure 1**. AF2's failure to predict whole antigen structure coupled with the scFv is highlighted in **Supplemental Figure 2**. Both the 'Simple Max' and 'Consensus' methods were calculated first by parsing every pLDDT score received by every residue in the antigen sequence sliding window output structures. From the resulting data structure, the Simple Max method simply finds the maximum pLDDT value ever seen for a single residue (across all sliding windows and AF2 models). For the Consensus method, per-residue pLDDT was first averaged across the 5 AF2 models. These averages are reported in the heatmap view and further averaged per sliding window for the bar chart below. In principle, the strategy

behind the Consensus method is to take into account agreement across the 5 AF2 models and provide insight into the confidence of entire epitopes (whole sliding windows of n=10 default) instead of disconnected, per-residue pLDDT maxima. Having two scoring metrics is useful because the selection of predicted hits can differ. As shown in **Figure 2**, part of the Myc epitope makes it into the top 5 peptides when selection is based on summing per-residue maximum pLDDT (despite there being no requirement that these values originate in the same physical prediction). In contrast, a Consensus method score more directly reports on a specific sliding window, and the strength of the highest confidence peptides is more directly revealed with superior signal to noise as shown in **Figure 3**. Variability in the ranking of top hits between the two methods arises from the fundamental difference in strategy (peptide-centric or residue-centric scoring) as well as close competition between the raw AF2 confidence in the known peptide and competing decoy sequences.

Testing of scFv:peptide structure prediction method using the Myc Epitope

We first tested the PAbFold method with the anti-Myc-scFv described in (38), using the full-length human Myc proto-oncogene protein sequence as the antigen. We initially used an antigen peptide length of 10 and a 1 amino acid sliding window. Given these parameters, the 9 a.a. Myc epitope motif (EQKLISEEDL) appeared intact within one of the 10-mer peptides, with subsets of the 8, 9, 11, and 12 a.a. appearing in neighboring sliding peptide windows. PAbFold generated predicted structures, each of which took an average of ~200 seconds to process. The entire process took approximately 12 hours on our GPU server. AlphaFold2 placed all peptides into or near the traditional antigen binding site between the CDR loops (**Supplemental Figure 3**). The average confidence (mean pLDDT across residues) for these peptides ranged from 20 to 90. When we inspect the consensus confidence for each residue in each sliding window (**Figure 2A**), the expected Myc peptide epitope (EQKLISEEDL) was one of several peptides with high average pLDDT. The second highest ranked peptide in this analysis (QKLISEEDLL) was a near perfect match for the expected epitope. We consider this window to be a successful prediction. Perhaps surprisingly, the peptide window with the exact match (EQKLISEEDL) did not score particularly well due to its average pLDDT of 51.0. In this instance, the expected epitope sequence did not stand out when plotting the maximum observed per-residue pLDDT for each residue (**Figure 2A, bottom**).

We proceeded to test predictions with two engineered scFv chimeras where loop grafting was used to place the Myc recognition CDRs onto two antibody framework regions with high *in vivo* performance, generating Myc-15F11 and Myc-2E2 scFv sequences. Epitope prediction performance was markedly improved with the chimeric scFvs (**Figure 2B** and **2C**). Specifically, the QKLISEEDLL peptide window became the top ranked peptide on the basis of average consensus pLDDT. In the case of Myc-2E2 (**Figure 2C**), the average confidence for the correctly predicted epitope was particularly high compared to alternate peptide windows, and another close match to the expected epitope (EEQKLISEED) was ranked within the top 5 peptides (**Figure 2D**). Ranking epitopes using the Simple Max analysis was similar; the region containing the correct epitope was nearly top ranked for Myc-15F11 and was top ranked for Myc-2E2 (**Figure 2E**). Thus, AlphaFold2 was able to more clearly detect authentic Myc antibody epitope using CDRs loop grafted onto the 2E2 or 15F11 frameworks, relative to the native Myc scFv framework.

To investigate the superior epitope recognition performance of the chimeric Myc scFvs, we aligned the Cα coordinates for the predicted scFv structures (predicted with and without the target epitope) to the reference crystal structure and calculated the RMSD for all backbone positions (N, Cα, C, O) and the loops (**Supplemental Figure 4**). Notably, regardless of the Myc scFv variant, the CDR loop RMSD improved by more than 1Å when the epitope was present. Secondly, consistent with the improved epitope prediction performance for the chimeric scFvs (15F11 and 2E2), the

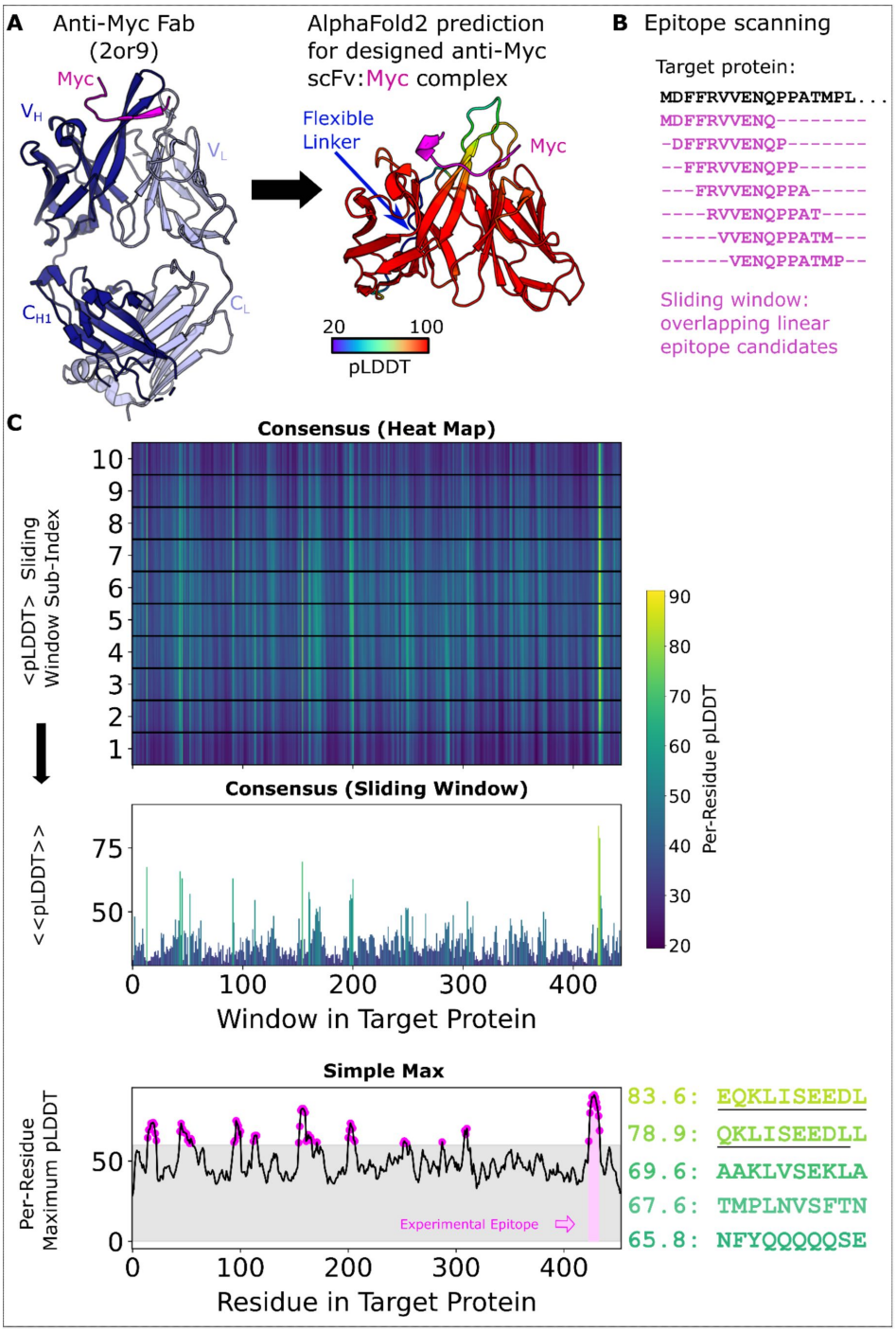


Figure 1.

PAbFold pipeline for linear epitope prediction.

A) Antibody V_H and V_L protein sequences are used to generate scFv sequences, either based on the native antibody sequences or loop grafting complementarity determining regions (CDRs) onto either the 2E2 or 15F11 antibody framework regions (2E2 shown). **B)** The target antigen sequence is parsed into a list of small overlapping peptide sequences, with peptide step and window size parameters adjusted as needed. Rank ordered peptides are output, and partial epitope sequences are underlined manually to highlight the identification of the correct sequence. **C)** The scFv sequences from Panel A are co-folded with each of the peptide sequences derived from the target antigen in parallel batch mode on a GPU server. pLDDT scores from each structure prediction experiment are collected and scores are presented in their sliding window, both as a heat map organized along the length of the target antigen sequence and a bar chart that shows the per-peptide average pLDDT (Consensus Method). Additionally, the Simple Max data is presented in the third and final panel.

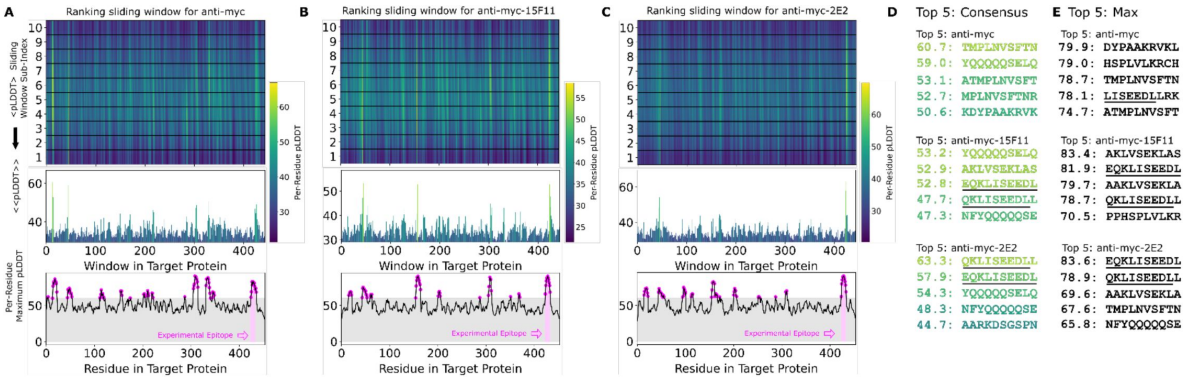


Figure 2.

The Alphafold2-based PAbFold method predicted the Myc linear epitope in different scFv backbones.

The anti-Myc V_H and V_L antibody sequences were used to generate either **A)** wild-type Myc scFv or loop grafted chimeric **B)** Myc-15F11 or **C)** Myc-2E2 scFv variants. The Myc proto-oncogene protein sequence (Genbank NP_001341799.1) was used as the target antigen and processed into 10 amino acid overlapping peptides with a 1 amino acid sliding window. The structure for each scFv:peptide pair was predicted with AlphaFold2 in batch mode on two NVIDIA A5000 GPUs. Average consensus pLDDT values for each scFv:peptide window are illustrated, as well as the maximum pLDDT observed for each residue in any window (bottom). **D)** Top ranking binding peptides based on average consensus pLDDT. **E)** Top ranked binding peptides based on summing per-residue maximum pLDDT. For D and E, underlining represents overlap with the reported Myc epitope (EQKLISEEDL).

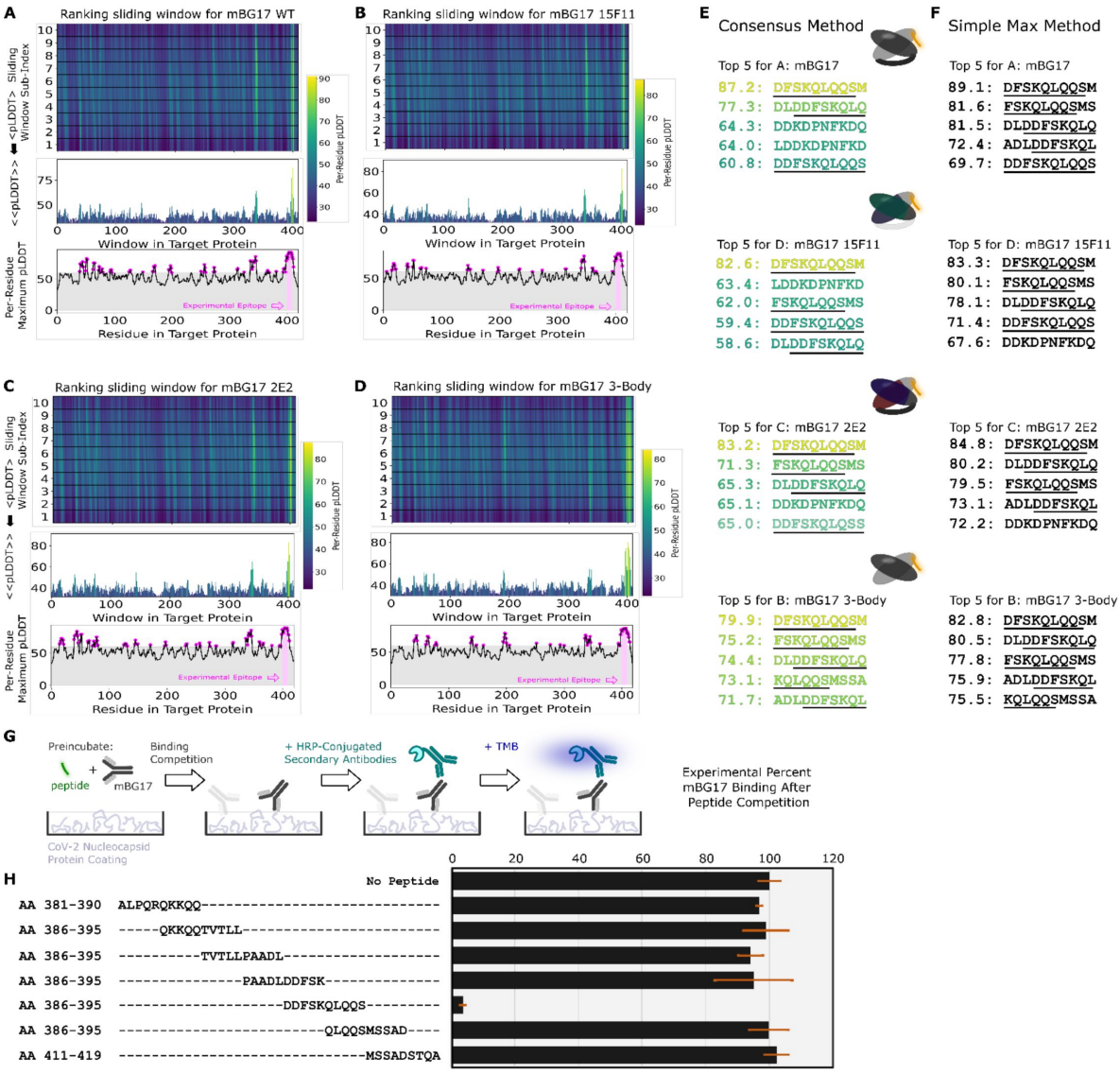


Figure 3

The AlphaFold2-driven PAbFold epitope scan method can accurately identify a linear epitope for a novel SARS-CoV-2 antibody.

Antibody VH and VL sequences for SARS-CoV-2 nucleocapsid protein targeted antibody were used to generate scFv sequences **A)** WT, **B)** 15F11, **C)** 2E2 or native VH and VL sequences **D)** 3 body). Variant scFv sequence in complex with peptide windows from the SARS-CoV-2 nucleocapsid protein (Genbank Accession: YP_009724397) were subjected to AlphaFold2 structure prediction. The top 5 peptides ranked by either the **E)** Consensus method or the **F)** Simple Max method, with the underlined sequence highlighting the experimentally verified sequences and a cartoon schematic for each system shown. **G)** Competition ELISA schematic for assessing the ability of synthetic peptides derived from the SARS-CoV-2 nucleocapsid protein. **H)** Amino acid windows showing binding interference, with mBG17 binding to SARS-CoV-2 nucleocapsid protein (n = 3). Percentage of binding values were calculated from the no-peptide control. Alignment of synthetic peptides corresponding to SARS-CoV-2 nucleocapsid a. a. 381-419. Peptide a. a. 401-410, which demonstrated mBG17 competition.

epitope peptide QKLISEEDL was placed more accurately for those predicted structures than in the WT scFv (**Supplemental Figure 4**). We could not discern an obvious structural difference between the WT and chimeric scFvs that explains the structure prediction performance gap.

Assessment of peptide length, sliding window size, and position on AlphaFold2 scFv:peptide structure prediction

Our initial selection of the 10 a.a. window was intended to match or slightly exceed the size of known epitopes such as Myc and HA. We next assessed how different peptide sizes and sliding window lengths would affect epitope prediction accuracy and run time. We re-ran the Myc-2E2-scFv:peptide complex prediction calculations varying peptide size between 8, 9, 10, and 11 (with a fixed sliding window size of 2) or varying the sliding window size to 1 or 5 (with a fixed peptide size of 10). We observed that using a sliding window of 2 a.a. provided nearly the same level of accuracy and resolution as the 1 a.a. Ultimately, we determined that our original peptide size of 10 amino acids and sliding window of 1 a.a. provided highest resolution data possible (**Supplemental Figure 5**) and therefore maintained a peptide size of 10 and a sliding window length of 1 for our remaining experiments.

We then predicted the complex structure for Myc-2E2 with various negative control peptides: A10, (GS)5, (GGGGS)2, and G10 to determine how non-binding peptides are docked and scored (**Supplemental Figure 5I** and **5J**). We again observed that AlphaFold2 placed all peptides into the traditional antigen binding between the CDR loops, but the reported peptide scores for the negative controls were particularly low (29–41). These results indicate that AlphaFold2 "knows" where antigens bind in antibody or scFv structures and attempts to model any peptide partner into this region, but the low pLDDT scores indicate confidence in the interactions are quite low.

We also tested if AlphaFold2 could detect the Myc epitope if it was inserted as an epitope tag within different positions of a heterologous protein. We created a synthetic antigen by adding the Myc epitope within the 99-a.a. unrelated HIV-1 Gag protease protein sequence at either the N- or C-terminus or in the middle of the protein sequence, and used PAbFold to detect the Myc peptide (**Supplemental Figure 6**). In each case, the average consensus pLDDT was highest for the inserted epitope, such that the authentic epitope would be top ranked and prioritized for testing. Thus, as expected for a sliding window analysis, the epitope position within the antigen was no barrier to detection.

Testing of the PAbFold method using the HA Epitope

Based on our success detecting the Myc epitope, we sought to determine if our method could detect a different well-known linear peptide, HA, derived from positions 114-126 within the Influenza A virus hemagglutinin protein (YDVPDYASLR). Using an anti-HA scFv sequence that had been previously generated (22, 38), we generated new HA-15F11 and HA-2E2 scFvs loop grafted sequences. We used the same procedure described above to predict structures for influenza A virus HA derived peptides on HA-scFv (**Supplemental Figure 7A**), HA-15F11-scFv (**Supplemental Figure 7B**) and HA-2E2-scFv (**Supplemental Figure 7C**). In the HA case, the expected epitope was ranked highly for all three scFv variants, but when assessing entire peptides by average consensus pLDDT was only ranked in the top 5 for the HA-15F11-scFv. These results, in combination with the Myc results described above, indicate that AlphaFold2 can accurately detect linear antibody epitopes in antigen sequences, and that grafting CDR loops onto alternative scFv backbones may increase the noise-to-signal ratio, making the identification of correct epitopes more accurate.

Like the Myc system, trends are observed with the HA system regarding loop placement. Although not as extreme, the loops for all HA scFvs undergo movement that make it more closely match the crystal structure (PDB entry 1frg). Again, the epitope placement of predicted structures of the

chimeric scFvs more closely mimicked the deposited crystal structure than the WT scFv (**Supplemental Figure 4B**).

Determination and experimental validation of a novel linear antibody epitope

The Myc and HA monoclonal antibodies are well known and several crystal structures (Myc PDB: 2or9, peptide bound (2009) | HA PDB:1frg, peptide bound (1994)) have been solved (22, 36, 38, 39), raising the possibility that AlphaFold2 has incorporated these antibody or epitope structures into its training set. The AlphaFold2 training set was reported to exclude chains of less than 10, which would eliminate the myc and HA epitope peptides. Nonetheless, to guard against the possibility that the AlphaFold2 models have incorporated specific knowledge into the training set thereby directly probing if PAbFold epitope scanning can predict a linear antibody epitope without *a priori* knowledge of the antibody or antigen sequence, we tested if PAbFold can predict the epitope sequence of a recently developed antibody lacking structural information available in the Protein Data Bank. The mBG17 mouse monoclonal antibody was generated in response to the COVID-19 pandemic, the antibody VH and VL sequences were determined, and the epitope was localized to a. a. 381-419 via Western blot analysis of deletion mutants of the nucleocapsid protein (34). mBG17 was not included in AlphaFold2's training or test set, making it an ideal test case for *de novo* epitope prediction.

The mBG17 monoclonal antibody was converted to wild-type scFv, 15F11-scFv, and 2E2-scFv using the same procedures used for Myc and HA scFv. As an additional control calculation (labeled "3-body"), we used AlphaFold2 to predict the structure for a 3-protein complex (the peptide, and the disconnected nontruncated mBG17 VH and VL variable domain sequences). All 4 Fab variants (WT scFv mBG17, 15F11-mBG17 scFv, 2E2-mBG17 scFv, and 3-body mBG17) were screened against all 10 a.a. peptides with a 1 a.a. sliding window, as with Myc and HA. In all 4 cases, AlphaFold2 predicted that the top ranked peptides were located in the a.a. 381-419 region of the SARS-CoV-2 nucleocapsid protein, and more specifically residues a.a. 400-415 (**Figure 3A**, **3B**, **3C**, and **3D**). The top scoring peptide for all three scFv variants was the 402-411 window (DFSKQLQQSM) (**Figure 3E** and **3F**). The strong AF2 preference for peptides from this C-terminal segment was particularly evident in the average consensus pLDDT analysis.

We next sought to experimentally verify the minimal linear epitope for mBG17 to determine how closely the AlphaFold2 prediction corresponded to our experimental data. Seven 10 a.a. peptides that overlapped by 5 a.a. each were synthesized and used in competition ELISAs with mBG17 monoclonal antibody and recombinant SARS-CoV-2 nucleocapsid protein (**Figure 3G** and **3H**). The peptide corresponding to a.a. 401-410 showed almost complete competition of mBG17 binding to the SARS-CoV-2 nucleocapsid protein in the ELISA, whereas none of the other peptides were able to compete for mBG17 binding to nucleocapsid. Peptides a.a. 296-405 and a.a. 406-415 overlap a.a. 401-410 at the N- and C-terminus, respectively, but neither was able to compete, indicating that mBG17 binds a.a. 401-410 on both sides of a.a. 405 and a.a. 406. An alignment of all the peptides used in the overlapping peptide competition ELISA experiments showed that peptide sequence DDFSKQLQQS represents the experimentally determined epitope for mBG17, nearly identical to the epitope predicted by AlphaFold2 (**Figure 3H**: DDFSKQLQQS). These results demonstrate that the PAbFold pipeline was able to very accurately predict the region that an antibody binds to a novel linear epitope that is not present in AlphaFold2's training set.

Fine-characterization of the mBG17 epitope and comparison to the predicted AlphaFold2 model

To further experimentally characterize the binding of the mBG17 to the a.a. 401-410 (DDFSKQLQQS) peptide and compare experimental data with the predicted AlphaFold2 model, we designed and synthesized ten additional peptides, each containing an alanine point mutation at

one position in the a.a. 401-410 peptide. The peptides are labeled D1A, D2A, F3A, S4A, K5A, Q6A, L7A, Q8A, Q9A, and S10A. Competition ELISAs were performed using increasing concentrations of each peptide to better assess differential binding (**Figure 4A**). As expected, WT (a.a. 401-410) peptide showed strong competition, although Q9A showed slightly better competition. This could be attributed to alanine's propensity to be in an alpha-helical coil (PropA, AHC = 0) vs glutamine's propensity to escape it (PropQ, AHC = 0.39) (40), thus further stabilizing the Q9A alpha helix. D1A showed no change in competition, indicating that D1 was not involved in binding. Peptides with substitutions K5A, Q6A, and S10A showed minor reductions in competition, S4A showed a moderate reduction on competition, whereas resides D2A, F3A, L7A, and Q8A all showed strong reductions in competition. These data indicate that the key interactions between mBG17 and the a.a. 401-410 peptide are residues D2, F3, L7, and Q8, with S4 playing a moderate role and D1, K5, Q6, Q9 and S10 playing negligible roles in binding.

Finally, we compared the experimental data shown above with the best scoring mBG17:DDFSKQLQQ model generated by AlphaFold2 (**Figure 4B** and **4C**). The AlphaFold2 model suggests that residue D2 forms a hydrogen bond with mBG17 a.a. Y34, residue F3 forms a hydrophobic interaction with mBG17 a.a. L185, residue S4 lacks a hydrogen bond partner, residue L7 forms a hydrophobic interaction at the base of the binding cleft with mBG17 a.a. A104, and residue Q8 hydrogen bonds with the backbone carbonyl of Y34 and the backbone amide of W35. Residues that experimentally showed no or minimal effects on competition (D1, K5, Q6, Q9) are all predicted to interact primarily with the solvent and lacked visible interactions between the peptide and scFv sequence. In summary, the AlphaFold2-driven PAbFold prediction was remarkably consistent with the experimental alanine scanning data, suggesting that the prediction of the mBG17 linear epitope location was accurate due to the correct prediction of the structural details for how that linear epitope binds to the antibody.

Discussion

In this project we assessed the ability of an AlphaFold2-based linear epitope scan pipeline we call PAbFold (Peptide:Antibody Fold) to predict linear antibody epitopes using just antibody and antigen sequences. We first assessed the quality of scFv models produced by AlphaFold2. We then developed a series of Python scripts that accept scFv and whole antigen protein sequences as inputs, parse the antigen protein sequences into short overlapping peptides, run batch predictions for each scFv:peptide pair, and output two peptide scoring schemes based on the peptide per-residue pLDDT scores as a metric for AlphaFold2 model confidence.

Binding of the expected epitope to the WT-Myc scFv could only be detected via the consensus method, but either analysis method could readily detect the expected epitope bound to the chimeric Myc scFvs. Conversely, the alternate analysis method (Simple Max) performed better with respect to ranking the expected HA epitope binding to the WT and chimeric anti-HA scFv variants. In the HA case, performance was comparable for both the WT and chimeric scFv variants.

It is important to note that binding of scFv variants to sequences other than the expected epitopes may be statistically unlikely but not impossible. For example, consider the peptide ATMPLNVSFT near the N-terminus of the Myc proto-oncogene protein sequence. In the context of the WT anti-Myc scFv this peptide had slightly higher average consensus pLDDT (52.4 rather than 51.0) than a peptide (QKLISEEDLL) that closely matched the expected epitope. In the absence of direct experimental evidence, predicted affinity for this unexpected sequence is not necessarily incorrect, though the lack of comparable predicted binding to the 15F11 and 2E2 chimeric scFv variants further decreases the likelihood. In the future, it might be useful to assess peptide binding via consensus across scFv variants.

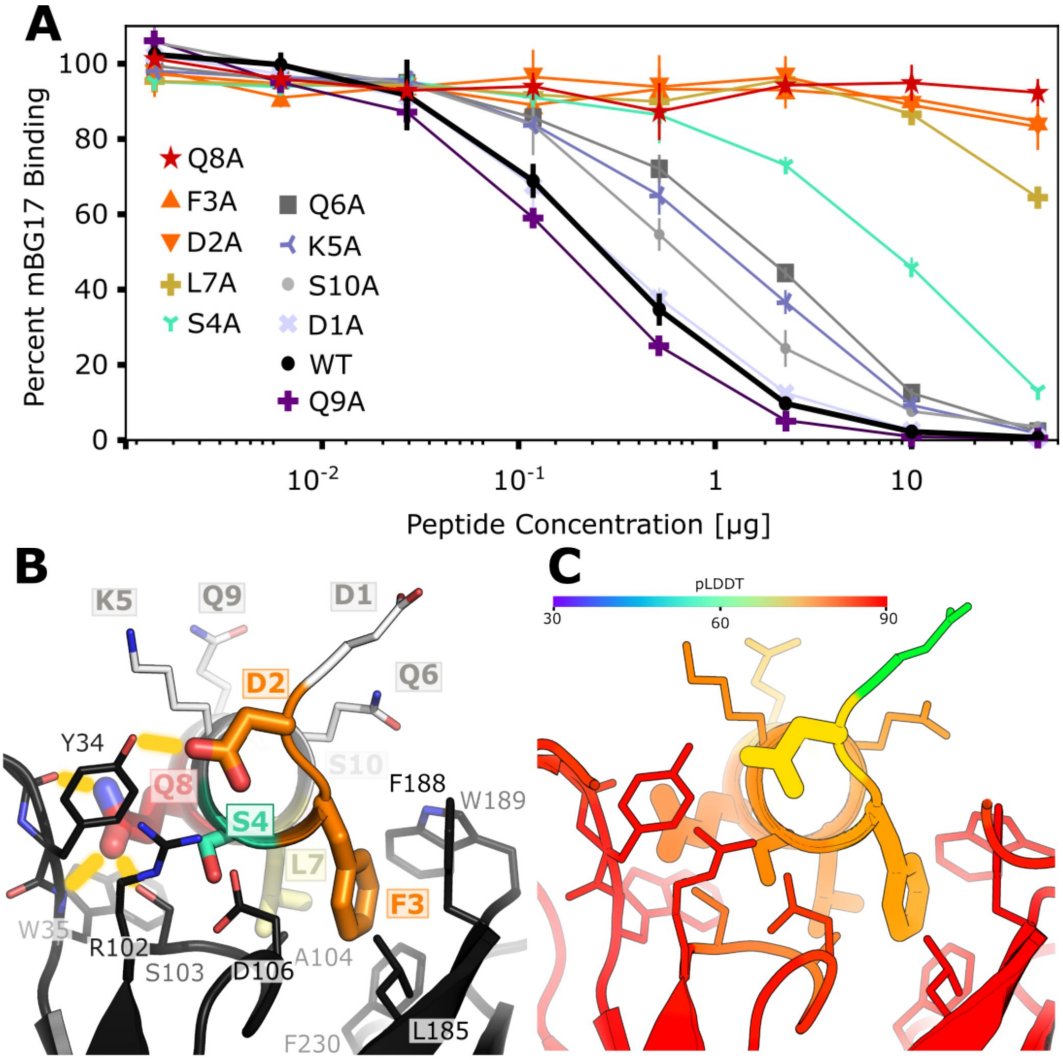


Figure 4.

The Alphafold2-Driven PAbFold method accurately predicts molecular interactions between a linear epitope and a scFv

A) Competition ELISA assessing the ability of synthetic alanine mutant peptides derived from the SARS-CoV-2 nucleocapsid protein (a. a. 401-410: DDFSKQLQQS) to interfere with mBG17 binding to SARS-CoV-2 nucleocapsid protein (n = 3). Percentage of binding values were calculated from the no-peptide control. **B)** AlphaFold2 model for mBG17-15F11 scFv bound to a. a. 401-410 peptide (the average peptide pLDDT was 83.5). Residues that display sharply reduced binding to mBG17 upon mutation to alanine in competition ELISAs (D2, F3, S4, L7, Q8) are shown as warm-colored thick sticks. Predicted hydrogen bonds between the peptide and the scFv are depicted by yellow bars. Sites where mutation to alanine was less disruptive to binding (Q6A, K5A, S10A, D1A, and Q9A) are depicted as thin sticks with cool colors. The carbon atoms of residues in panel B are colored according to the corresponding data in panel A. **C)** The same AlphaFold2 model for the mBG17-15F11 scFv bound to a.a. 401-410 colored with confidence (pLDDT) as predicted by AF2.

Lastly, we tested this process on a novel antibody generated by our group targeting the SARS-CoV-2 nucleocapsid protein (mBG17) and found the method performed significantly better than with Myc and HA. Either analysis method could very easily flag peptide windows containing the authentic experimentally validated epitope. This worked for the WT scFv, the chimeric scFv variants, and even a structure with disconnected heavy and light chain domains. Experimentally, we cleanly validated the AlphaFold2 prediction using a peptide competition ELISA assay to experimentally determine the mBG17 epitope. Confidence in the AlphaFold2 prediction was further buoyed via alanine scanning peptide competition ELISAs that verified the importance of the key binding interactions predicted by AlphaFold2.

Identification of antibody VH and VL sequences from monoclonal B-cells has become a routine task, with sequence information obtainable via various sequencing technologies such as next generation sequencing and nanopore sequencing for a relatively low cost. As a result, the determination of the epitope in service of a deeper understanding of how antibodies bind their antigen is an increasingly notable bottleneck. An experimental epitope determination campaign can take weeks or months of work, but with the advent of AlphaFold2 and the epitope prediction method we describe here, an antibody and its antigen could be sequenced in a few days (often through contract research organizations for low cost) and accurate linear epitope predictions generated within less than a day, dramatically epitope validation throughput as well as providing detailed predictions for the molecular features of antibody-epitope interaction.

Conformational epitopes are structured antigens that are found during many immune responses, and prediction of these epitopes from antibody and antigen sequences would be a significant boon to the field of biology. For example, conformational epitope prediction coupled with single-cell B-cell sequencing would allow for detailed analysis of antibody maturation during immune responses to vaccines or pathogen infection, helping better define how the immune response to infection evolves over time and how evolution of antigen sequences affects the antibody response. In this work we did not focus on using AlphaFold2 to predict conformational epitopes primarily because of the complex structures that conformational epitopes possess. Literature reports suggest that prediction of the complexes between antibodies and both whole antigens and conformational epitope proteins has proven to be very difficult for AlphaFold2, and indeed the authors themselves make this observation (12, 41, 42). Notably, the structures that proved most difficult to predict for AF2 and other tools in the CASP15-CAPRI154 challenges were antibody-antigen complexes (43). Reports suggest that a mix of both statistics-based approaches (neural networks like AF2) and physics-based approaches (such as Rosetta) predict optimal antibody-antigen complexes (44). Indeed, if we attempt to predict binding of our scFvs to intact antigen proteins (**Supplemental Figure 2**), we find no predictive capability. When predicting scFv:peptide complexes, it may be the case that AlphaFold2 is able to thoroughly evaluate an induced fit for the peptide due to both its length (small sample space) and its propensity to not adopt a strong competing structure. In contrast, embedding the epitope within a larger and more complicated structure appears to degrade the ability of AlphaFold2 to sample a comparable bound structure within the allotted recycle steps. Additional complexities may arise in extreme induced conformational changes during docking. Recent reports indicate that progress is being made in predicting the binding locations of conformational epitopes (45, 46).

We observed that the ability of AlphaFold2 to successfully predict the epitope peptide binding is quite delicate. First, epitope prediction was highly sensitive to the peptide length (**Supplemental Figure 5**), with minimal predictive power for peptide length other than 10 a.a. Further investigation of this sensitivity would be a useful avenue for future research. Perhaps with enhanced sampling, epitopes can be detected within longer peptides (e.g. 11 a.a., 12 a.a., etc.). Methodological tuning of this type could ultimately help illuminate the path to increasingly difficult protein-protein binding prediction problems. Similarly, we have likewise determined that epitope scanning performance was sensitive to changes in the underlying AlphaFold2 neural networks and the MSA. Specifically, unless otherwise noted, all data in this report was obtained

using ColabFold version 1.5.2 and the 5 neural networks that comprise AlphaFold2 multimer version 2 (mm2). Likewise, the MSAs we use were obtained from the MMSEQS server (and cached) when the default sequence databases were UniRef30 2202 and PDB70 220313. They have since been updated to PDB30 2302 and PDB100 230517. For a complete description, see the change logs on the github for ColabFold (https://github.com/sokrypton/ColabFold#colabfold---v152).

Insofar as protein-peptide prediction is an emergent "off-label" capability for AlphaFold2 that is not part of the training sets, further training of the models or other changes can degrade performance. Benchmarking performance can be difficult when there are multiple moving targets. The most recent calculations we have analyzed were using ColabFold version 1.5.2 which was current as of February 19, 2023. The changes from ColabFold 1.5.2 to 1.5.5 (current as of this writing) are limited to version control and ensuring ColabFold still works on Google Colab and therefore will not change the calculation performance. Relative to ColabFold 1.3 (the current method at the outset of this project), ColabFold 1.5.2 embodied two substantial changes. First, ColabFold 1.5.2 used the updated AlphaFold multimer (mm) version 3 by default. Second, the backend server MMSEQS ((47) and (*https://github.com/soedinglab/MMseqs2*)) that supplies MSAs also underwent updates, namely the database updates. Upon evaluation, we found that the recent default methods (ColabFold 1.5.2) still predicted the epitope successfully for the mBG17 system (**Supplemental Figure 8**). However, the ColabFold 1.5.2 default methods had a pronounced decline in PAbFold performance for the HA and Myc systems. Specifically, the combination of mm3 and the revamped ColabFold MSA server tended to be less discriminating compared to the default settings for ColabFold 1.3 (ColabFold 1.3 was the most up to date version when this project was initialized). The updated configuration flagged diverse peptide sequences with elevated pLDDT values (**Supplemental Figures 9** and **10**) resulting in the loss of successful epitope predictive power. While testing ColabFold 1.5.2 with the most recent MSA server, but reverting the AlphaFold2 models to mm2, the outcome improved, with experimentally validated sequences rising to the top more frequently than when using mm3 but still falling short in ranking the experimentally validated epitope sequence embedded within the antigen. However, when previously cached MSAs were paired with mm2 (using ColabFold 1.5.2), performance was maximized. Furthermore, we attempted to recreate the MSA databases locally with similar but not identical results to queueing the server with databases UniRef30 2202 and PDB70 220313 (**Supplemental Figure 11**). Additionally, the MMSEQS team ((47) and (*https://github.com/soedinglab/MMseqs2*)) graciously rebuilt a server we could query using LocalColabFold that mimicked the original UniRef30 2202 and PDB70 220313 database set up as closely as possible on their end. The MSA that was generated from these databases was used, and still did not perform as well as the original MSAs that were generated upon first retrieval and generation (**Supplemental Figure 12**). As a negative control, we repeated all calculations without using any MSAs and only relying upon the sequence to make a structural prediction. As expected, all epitopes were scored very poorly (**Supplemental Figure 13**). Despite our significant efforts, it is unclear why our initial results cannot be perfectly recapitulated, but the difference has been traced to detailed MSA contents (**Supplemental Figure 14**), resulting in differences in correct epitope identification. These results are summarized in (**Supplemental Figure 15**). These challenges are presumably compounded by the incredible diversity of the CDR loops in antibodies which could decrease the useful signal from the MSA as well as drive inconsistent MSA-dependent performance

One key lesson of this research effort is that caching the MSAs proved to be very useful as a method to guard against changes in the performance of 3rd party tools. We recommend that future methods development work using LocalColabFold adopt the strategy of caching MSAs when feasible. It is also our hope that by describing the latent ability of AlphaFold2 to predict scFv-binding epitopes that this ability will be preserved and enhanced in future iterations.

Supplemental tables and figures

>mBG17 scFv
MAEVKLEESGGGLVQPGGSMKFSCVASGFTFSDYWMNWVRQSPDKGLEWVAEIRLKSNNYATHYAASVKGRFTISRDDSK
SSVYLQMNNLRAEDSGIYYCTRSAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVMSQSPSSLAVSVGEKITMSCKSS
QSLLYTSDQKNYLAWFQQKPGQSPKLLIFWASTRDSGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQFYNYPRTFGGGT
KLEI

>mBG17-15F11
MAEVKLVESGGGLVKPGGSLKLSCAASGFTFSDYWMNWVRQTPEKRLEWVAEIRLKSNNYATHYAASVKGRFTISRDNAK
NTLYLQMSSLRSEDTAIYYCARSAMDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVLTQSPASLTVSLGQRATISCKSSQSLL
YTSDQKNYLAWYQQKPGQPPKLLIYWASTRDSGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQFYNYPRTFGAGTKLEI

>mBG17-2E2
MAEVQLVESGGDLVKPGGSLKLSCAASGFTFSDYWMNWVRQTPDKRLEWVAEIRLKSNNYATHYAASVKGRFTISRDNAK
NTLYLQMSSLKSEDTAMYYCARSAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCKSSQS
LLYTSDQKNYLAWYQQKPGQPPKLLIYWASTRDSGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQFYNYPRTFGGGTKLE
I

>mBG17 Fab VH:VL
MYLGLNCVFIVFLLKGVQSEVKLEESGGGLVQPGGSMKFSCVASGFTFSDYWMNWVRQSPDKGLEWVAEIRLKSNNYATH
YAASVKGRFTISRDDSKSSVYLQMNNLRAEDSGIYYCTRSAMDYWGQGTSVTVSS:MDSQAQVLMLLLLWVSGTCGDIVM
SQSPSSLAVSVGEKITMSCKSSQSLLYTSDQKNYLAWFQQKPGQSPKLLIFWASTRDSGVPDRFTGSGS

>mBG17 epitope
DDFSKQLQQS

>mBG17 target protein sequence – SARS CoV-2 Nucleocapsid protein
MSDNGPQNQRNAPRITFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALTQHGKEDLKFPRGQGVPINTNSS
PDDQIGYYRRATRRIRGGDGKMKDLSPRWYFYYLGTGPEAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAAIVLQ
LPQGTTLPKGFYAEGSRGGSQASSRSSSRSRNSSRNSTPGSSRGTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQ
QQGQTVTKKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQGNFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSRI
GMEVTPSGTWLTYTGAIKLDDKDPNFKDQVILLNKHIDAYKTFPPTEPKKDKKKKADETQALPQRQKKQQTVTLLPAADLDD
FSKQLQQSMSSADSTQA

>HA scFv
MAEVKLVESGGDLVKPGGSLKLSCAASGFTFSSYGMSWVRQTPDKRLEWVATISRGGSYTYYPDSVKGRFTISRDNAKNTLY
LQMSSLKSEDTAMYYCARRETYDEKGFAYWGQGTTVTVSSGGGGSGGGGSGGGGSDIELTQSPSSLTVTAGEKVTMSCKSS
QSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGVPDRFTGSGSGRDFTLTISSVQAEDLAVYYCQNDNSHPLTFGAG
TKLEL

>HA-15F11

Supplemental Table 1A

Supplemental Table 1A (continued)

MEVKLVESGGGLVKPGGSLKLSCAASGFTFSSYGMSWVRQTPEKRLEWVATISRGGSYTYYPDSVKGRFTISRDNAKNTLYL
QMSSLRSEDTAIYYCARRETYDEKGFAYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVLTQSPASLTVSLGQRATISCKSSQSL
LNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQNDNSHPLTFGAGTKLEI

>HA-2E2
MAEVQLVESGGDLVKPGGSLKLSCAASGFTFSSYGMSWVRQTPDKRLEWVATISRGGSYTYYPDSVKGRFTISRDNAKNTLY
LQMSSLKSEDTAMYYCARRETYDEKGFAYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQRATISCKSS
QSLLNSGNQKNYLTWYQQKPGQPPKLLIYWASTRESGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQNDNSHPLTFGGGT
KLEI

>HA target protein sequence – influenza hemmaglutanin A
MKTIIALSYILCLVSAQKLPGSENRTATLCLGHHAVQNGTLVKTITNDQIEVTNATELVQSSSTGRICDNPHRVLDGRDCTLIDA
LLGDPHCDSFQNKEWDLFIERSKAYSNCYPYDVPDYASLRSLVASSGTLEFTTEGFDWTGVTQNGTSYSCKRGSANSFFSRLN
WLHKLNYKYPAQNVTMPNDDKFDKLYIWGVHHPSTDNDQTSLYVQTSGRVTVSTKRSQQTVVPDIGSRPWVRGISSRISIH
WTIVKPGDILLINSTGNLIAPRGYFKIRNGKSSIMKSDALIGNCNSECITPNGSIPNDKPFQNVNRITYGDCPRYVKQSTLKLAT
GMRNVPEKQTRGIFGAIAGFIENGWEGMVDGWYGFRHRNSEGTGQAADLKSTQAAIDQINGKLNRLIKKTNEKFHQIEKE
FSEVEGRIQDLEKYVEDTKVDLWSYNAELLVALENQHTIDLTDSEMNKLFERTRKQLRENAEDMGNGCFKIYHRCDNACIGS
IRNGTYNHNVYRDEALNNRFKIKGVELKSGYKDWILWISFAISCFLLCVGLMGLIMWTCQKGNIRCIRCNICH

>HA epitope
YPYDVPDYA

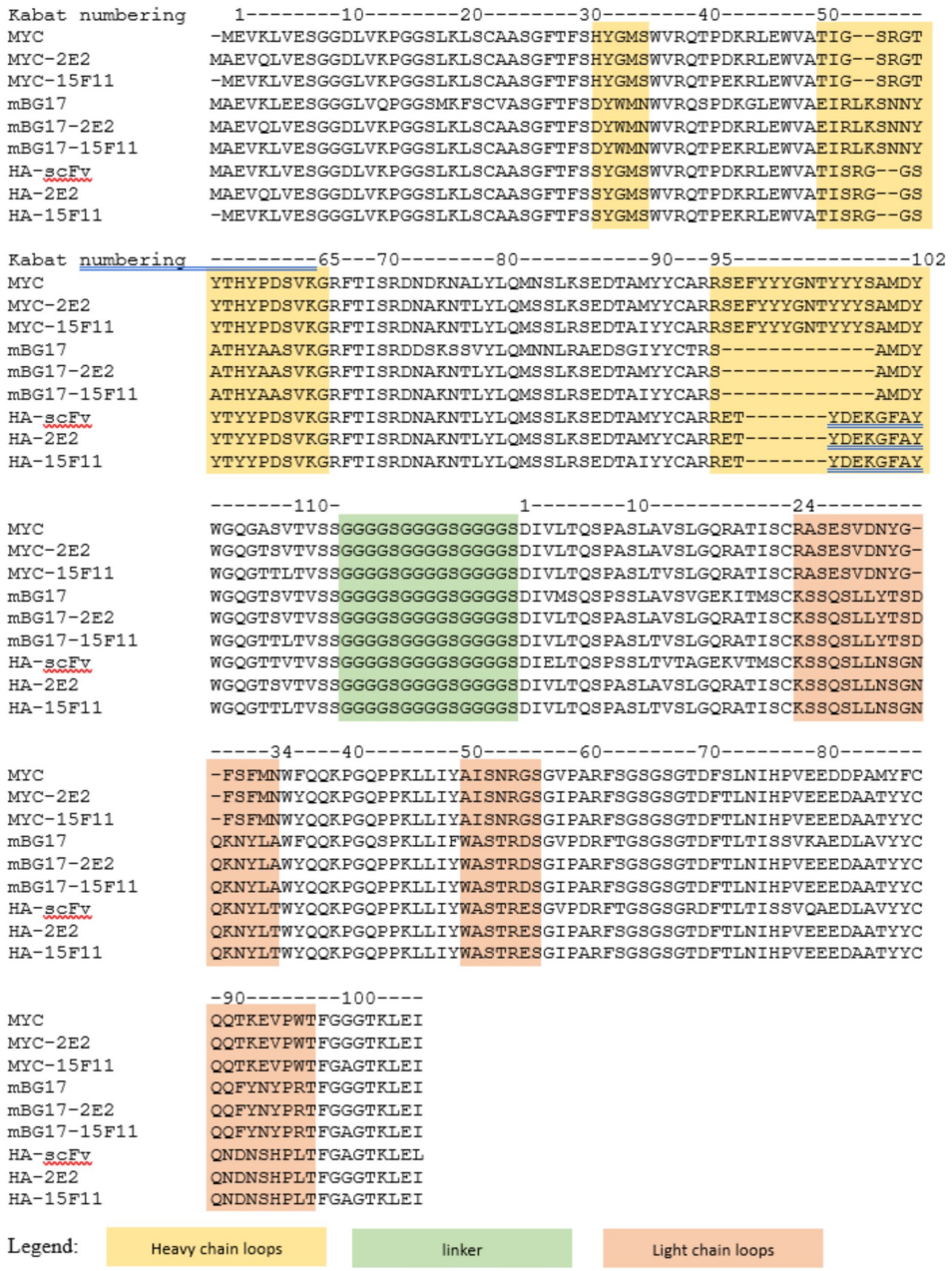
>Myc scFv
MEVKLVESGGDLVKPGGSLKLSCAASGFTFSHYGMSWVRQTPDKRLEWVATIGSRGTYTHYPDSVKGRFTISRDNDKNALY
LQMNSLKSEDTAMYYCARRSEFYYYGNTYYYSAMDYWGQGASVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQ
RATISCRASESVDNYGFSFMNWFQQKPGQPPKLLIYAISNRGSGVPARFSGSGSGTDFSLNIHPVEEDDPAMYFCQQTKEVP
WTFGGGTKLEI

>Myc-15F11
MEVKLVESGGGLVKPGGSLKLSCAASGFTFSHYGMSWVRQTPEKRLEWVATIGSRGTYTHYPDSVKGRFTISRDNAKNTLYL
QMSSLRSEDTAIYYCARRSEFYYYGNTYYYSAMDYWGQGTTLTVSSGGGGSGGGGSGGGGSDIVLTQSPASLTVSLGQRATI
SCRASESVDNYGFSFMNWYQQKPGQPPKLLIYAISNRGSGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQTKEVPWTFG
AGTKLEI

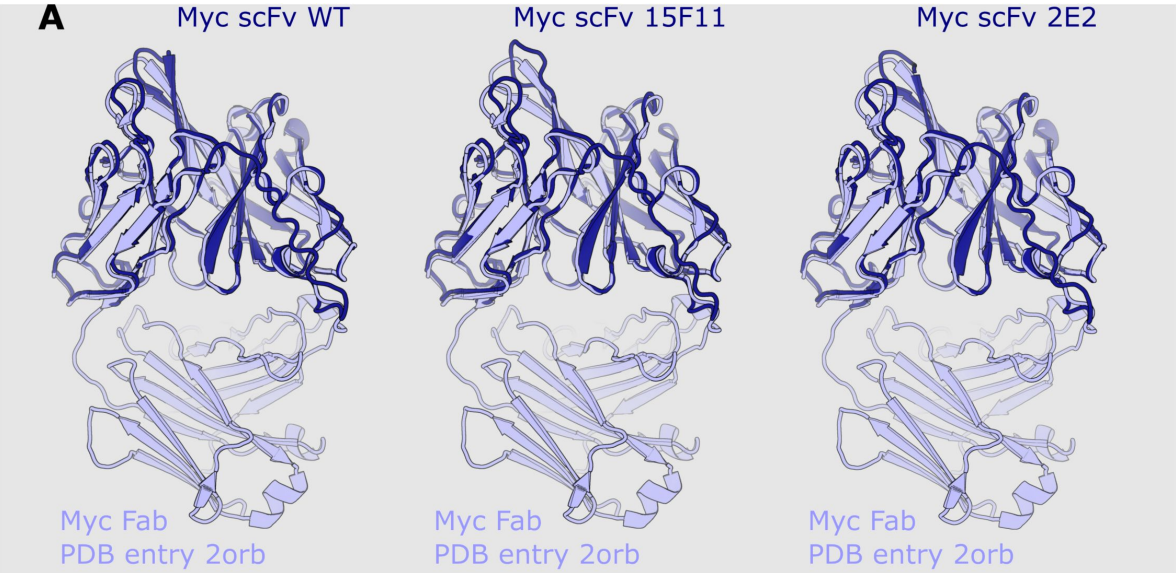
>Myc-2E2
MAEVQLVESGGDLVKPGGSLKLSCAASGFTFSHYGMSWVRQTPDKRLEWVATIGSRGTYTHYPDSVKGRFTISRDNAKNTL
YLQMSSLKSEDTAMYYCARRSEFYYYGNTYYYSAMDYWGQGTSVTVSSGGGGSGGGGSGGGGSDIVLTQSPASLAVSLGQ
RATISCRASESVDNYGFSFMNWYQQKPGQPPKLLIYAISNRGSGIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQTKEVP
WTFGGGTKLEI

>Myc target protein sequence
MDFFRVVENQPPATMPLNVSFTNRNYDLDYDSVQPYFYCDEEENFYQQQQQSELQPPAPSEDIWKKFELLPTPPLSPSRRS
GLCSPSYVAVTPFSLRGDNDGGGGSFSTADQLEMVTELLGGDMVNQSFICDPDDETFIKNIIIQDCMWSGFSAAAKLVSEKL
ASYQAARKDSGSPNPARGHSVCSTSSLYLQDLSAAASECIDPSVVFPYPLNDSSSPKSCASQDSSAFSPSSDSLLSSTESSPQGS
PEPLVLHEETPPTTSSDSEEEQEDEEEIDVVSVEKRQAPGKRSESGSPSAGGHSKPPHSPLVLKRCHVSTHQHNYAAPPSTRK
DYPAAKRVKLDSVRVLRQISNNRKCTSPRSSDTEENVKRRTHNVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATA
YILSVQAEEQKLISEEDLLRKRREQLKHKLEQLRNSCA

>Myc epitope
EQKLISEEDL



Supplemental Table 1B



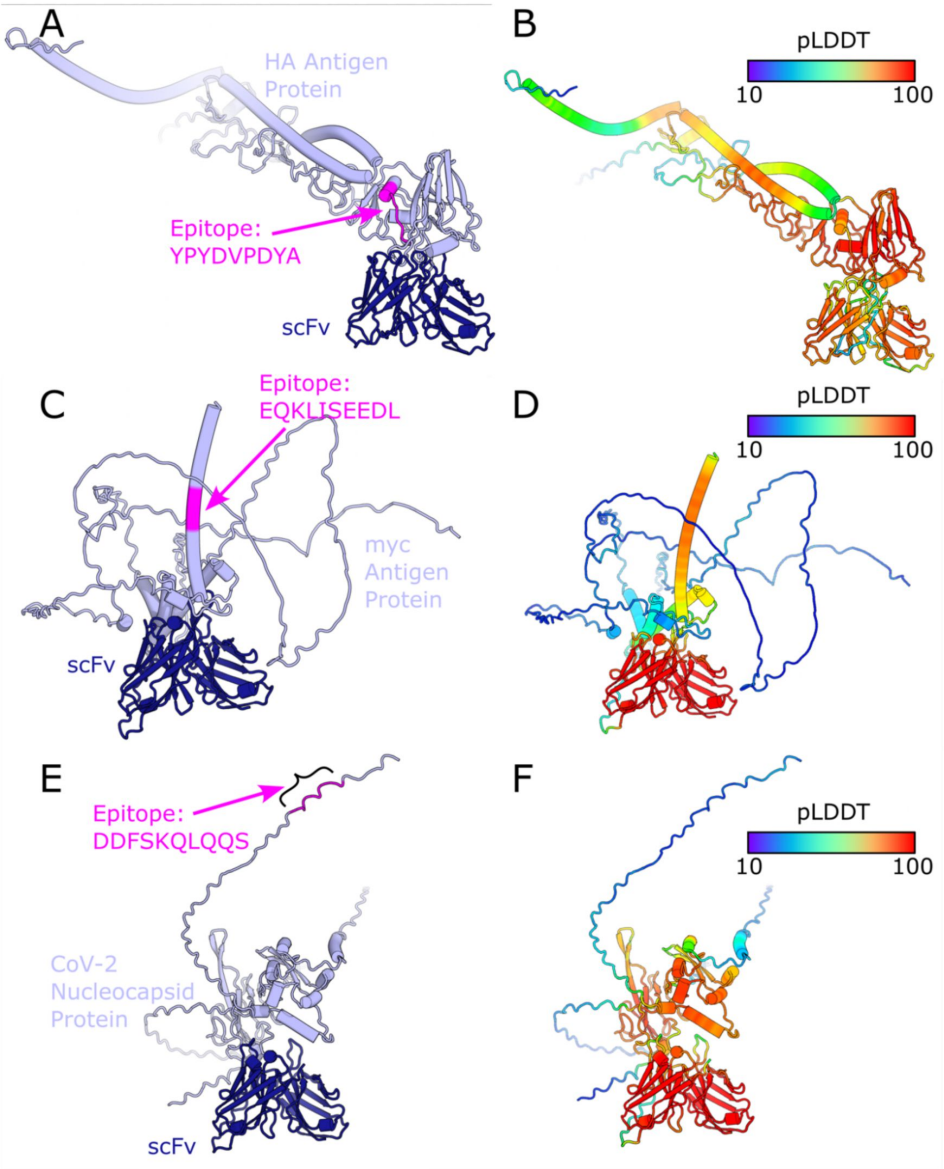
B

Structure	RMSD $_{Ca}$ [Å] vs 2orb
Myc scFv WT	0.42
Myc scFv 15F11	0.45
Myc scFv 2E2	0.45

Supplemental Figure 1.

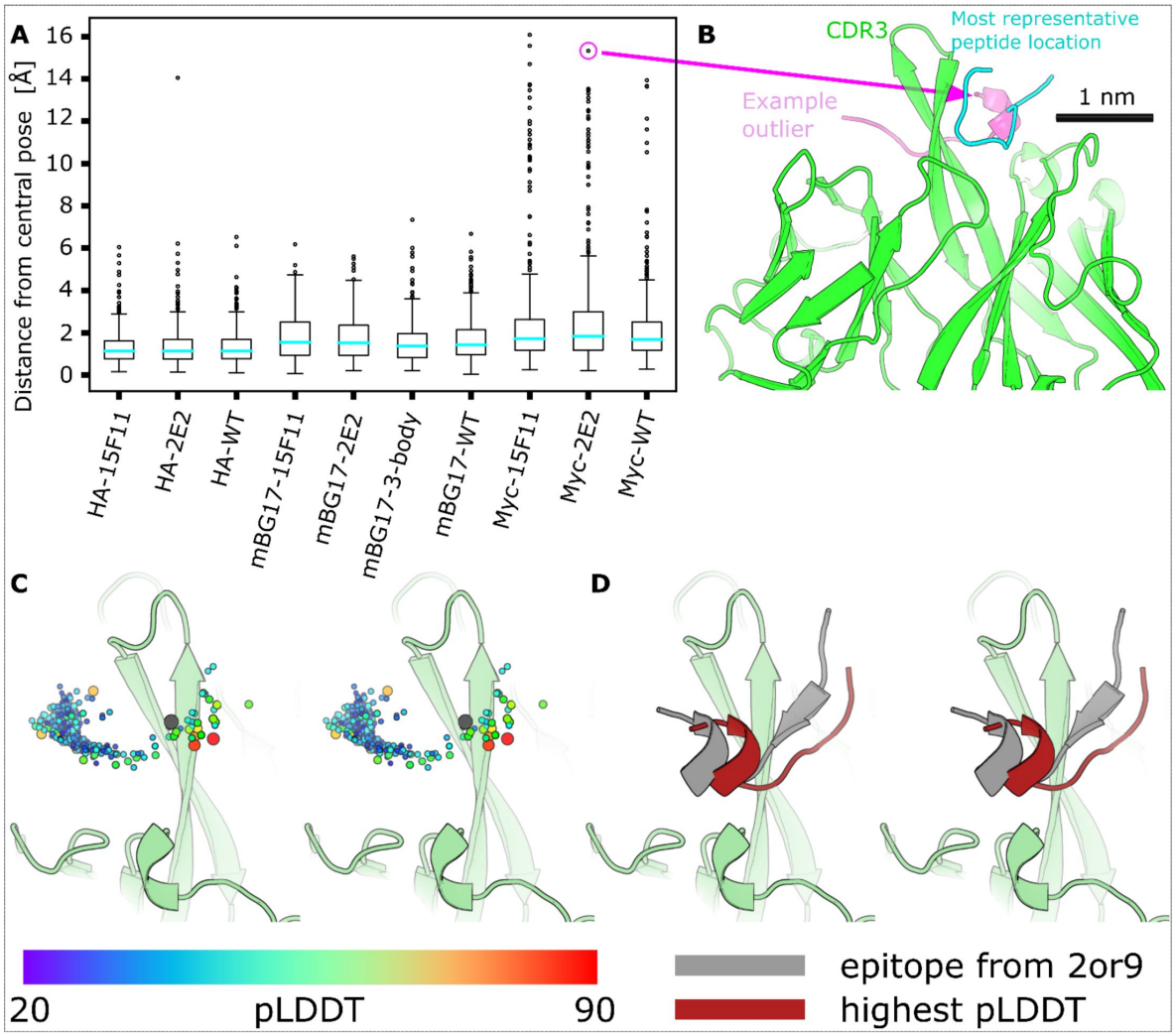
Alignment of AlphaFold2 predicted scFv structures to an anti-c-Myc Fab crystal structure.

A) Alignments of AlphaFold2-derived wild-type Myc scFv, Myc-2E2 scFv, and Myc-15F11 scFv structures with a Myc Fab crystal structure (PDB: 2orb). Predicted scFv structures are shown in dark blue, 2orb Myc Fab structures are shown in light blue. **B)** RMSD values comparing structural similarities between the wild-type Myc scFv, Myc-2E2 scFv, and Myc-15F11 scFv structures with a Myc Fab crystal structure (PDB: 2orb) were computed by the PyMOL align command.



Supplemental Figure 2

Alphafold2's best attempt to dock whole sequences with the respective sequence's scFv. **A)** The whole HA protein structure and scFv complex as predicted by AF2, with the correct epitope sequence highlighted in magenta. **B)** Shows the same structure by highlighted by confidence (pLDDT) of the structure with AF2. Similarly, the entire Myc protein-scFv complex are shown with **C)** the correct epitope highlighted in magenta and **D)** the confidence of the structure shown, and again for the mBG17 N-protein-scFv complex in **E)** and **F)**.



Supplemental Figure 3

AlphaFold2 places all peptides near the CDR loops.

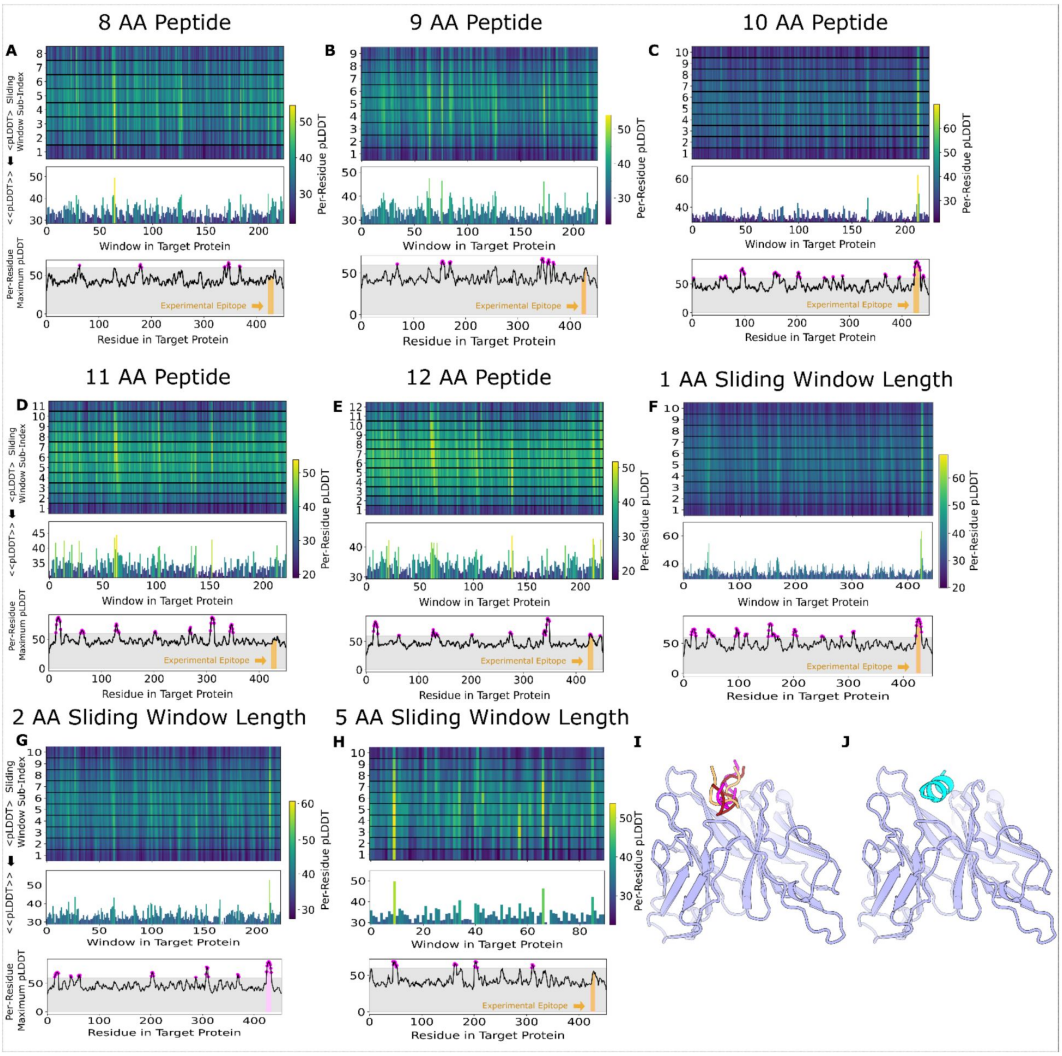
The predicted Cα coordinates for all scFv (excluding the flexible linker) were extracted, and all were aligned together using the Kabsch algorithm (48, 49). With the scFvs structurally aligned, an all-against-all RMSD was calculated for the epitope peptides. To visually represent each peptide as a single point, the coordinates for all epitope atoms were averaged. The "central" exemplar epitope (cyan) is the peptide with the smallest sum of RMSD to all other peptides. **A)** The average and quartile for peptide placement relative to the central peptide via Box-and-Whisker plot reveals that AlphaFold2 largely places all epitopes in the same area. The Myc CDRH3 runs through the middle of a traditional paratope pocket, it isn't a "cradle" for the epitope to sit on. AlphaFold2 places peptides on both sides of the CDRH3, causing significant spread in the peptide placement. **B)** An example of an exemplar, most-central predicted peptide structure (cyan) for the peptide PKSCASQDSS (cyan) bound to the Myc-2E2 scFv (green) that is distant from an example outlier peptide (magenta, peptide PHSPLVLKRC, center-to-center distance 14.8 Å). All peptide placements are still in contact with CDRH3, consistent with a strong AlphaFold2 bias to place peptides in a typical antibody binding site. **C)** The Myc-2E2 scFv (pale-green) and the average epitope placement (cyan) peptide alongside the crystal structure solution of the Myc epitope (grey). Remaining peptide placements are represented as a cloud of spheres at the mean peptide position. Each peptide sphere is colored and sized by epitope pLDDT (ranging from 20 to 90). Although AlphaFold2 frequently placed peptides on the opposite side of the CDRH3 from the Myc epitope (grey), it was not confident in these peptide placements (low, small, blue pLDDT spheres). In contrast, some of the peptides placed around the CDRH3, and in positions similar to the native epitope (grey) were placed with higher pLDDT confidence (increasingly large spheres trending from green to yellow to orange and red). **D)** The top ranked peptide as predicted by PAbFold with sequence QKLISEEDLL (red) and the crystal structure solution of the Myc epitope (grey).

scFv	Apo			Docked		
	BB Ca RMSD	Loop all backbone RMSD	Epitope all atom RMSD	BB Ca RMSD	Loop all backbone RMSD	Epitope all atom RMSD
Myc	0.65	2.87	NA	0.47	1.75	6.69
Myc-15F11	0.62	3.06	NA	0.51	1.51	2.45
Myc-2E2	0.61	2.96	NA	0.51	1.61	2.68

scFv	Apo			Docked		
	BB Ca RMSD	Loop all backbone RMSD	Epitope all atom RMSD	BB Ca RMSD	Loop all backbone RMSD	Epitope all atom RMSD
HA	0.56	1.39	NA	0.58	1.25	3.2
HA-15F11	0.56	1.32	NA	0.6	1.26	3.1
HA-2E2	0.58	1.21	NA	0.6	1.27	3.1

Supplemental Figure 4

RMSD comparison (all numbers have units of Å) for AlphaFold2 predicted scFv structures compared to reference crystal structures, **A)** 2or9 (Myc) and **B)** 1frg (HA), respectively. The loops of the scFv more closely mimic the crystal structure when the epitope peptide is present. The backbone also undergoes subtle changes during docking that make it slightly more similar to the crystal structure. These structures were aligned by identifying the framework residues in all structures, then aligning the framework region Cα with the Kabsch algorithm (48, 49). Specifically excluded from this process were the heavy and light CDR loops of the structures, as well as the flexible linker structure that connects the heavy and light chains due to the inherent floppy, unstructured nature of this region. After aligning the framework regions of the AlphaFold2 predicted structures and the crystal structures (2or9 and 1frg respectively), an RMSD of these Cα was calculated and is reported as the first column 'BB Cα RMSD'. Without further alignment, loop placement was analyzed with an all backbone RMSD by calculating the RMSD between the C, Cα, N, and O along the backbone of all residues in the scFv that were not used for the framework superimposition. This RMSD is reported in the second column as 'Loop all backbone RMSD'. Finally, to investigate peptide predicted placement and potential scFv:epitope interactions, an all-atom RMSD was calculated between the crystal structure and the AF2 predicted peptide structure (no additional alignment). Because the apo structure lacks a peptide position, this is only reported in the 'Docked' category and is in the 3rd column labeled 'Epitope all atom RMSD'. One script was written for each scFv (Myc and HA), and can be found in the Zenodo deposition of our data (*https://zenodo.org/records/10884181*) because this analysis is not a key part of PAbFold. Briefly this analysis reveals that all three HA scFv variants have predicted framework regions and loop regions in the apo structures that closely match the reference structure (0.56-0.58 Å and 1.21-1.39 Å). Accordingly, when the cognate epitope peptide is present, it can be placed with relatively high accuracy for all three scFvs (3.1-3.2 Å), with only small changes in the loops (1.39 Å to 1.25 Å, 1.32 Å to 1.26 Å, and 1.21 Å to 1.27 Å). In contrast, the apo structures for the three Myc scFvs have a much higher deviation in the loop regions (2.87 to 3.06 Å). When the epitope peptide is added, there is significant motion in the loops consistent with an "induced fit" description. In the two chimeric Myc scFvs (Myc-15F11 and Myc-2E2) the final loop RMSD is reduced to 1.51-1.61 Å, and the epitope peptide is successfully predicted (2.45-2.68 Å). However, despite a lower apo-state loop RMSD (2.87 Å), the loop RMSD for the wild-type Myc scFv only drops to 1.75 Å, and the epitope peptide placement does not match the experimental structure (6.69 Å). This is consistent with the failure of the wild-type Myc scFv AlphaFold2 predictions in main text **Figure 2**.



Supplemental Figure 5.

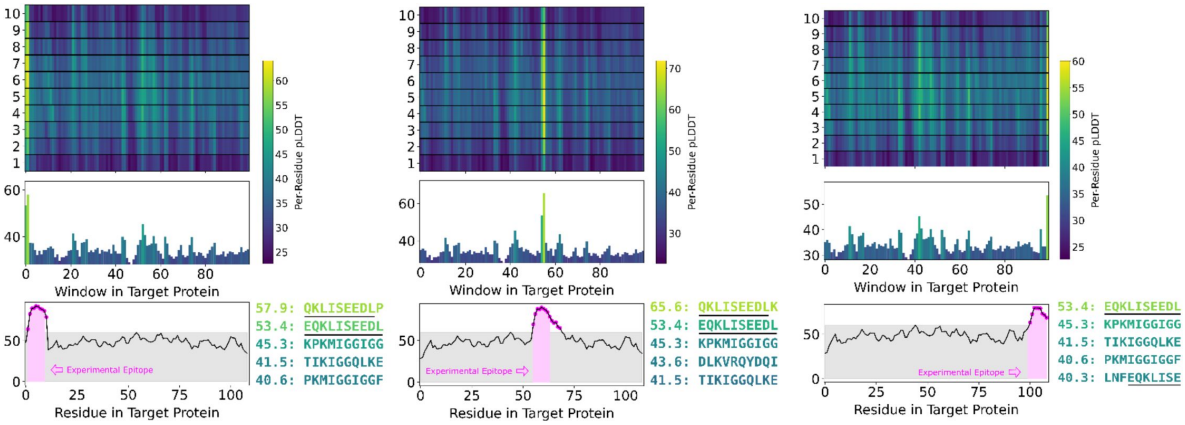
Assessment of peptide size and sliding window sizes on epitope prediction efficacy.

Myc-2E2 scFv:peptide structures were predicted with peptides of 8 (**A**), 9 (**B**), 10 (**C**), 11 (**D**), and 12 (**E**) amino acid lengths derived from the Myc protein with a sliding window of 2 amino acids, and pLDDT scores from each predicted structure were plotted against the Myc amino acid position and sliding window length target. **F)** Negative control peptides bind to antibody binding sites, but with poor pLDDT scores. Similarly, with a fixed peptide length of 10 and a sliding window step size of 1 (**F**), 2 (**G**), and 5 (**H**), we can see the practical epitope detection outcome was similar for a sliding window of 1 and 2, but resolution and accuracy were reduced for a sliding window step size of 5. To more fully illustrate the strong learned bias that AlphaFold2 has for placing any peptides among the CDR loops, we predicted the structure of Myc-2E2 in complex with several control peptides. These negative control peptides bind to the generally expected antibody binding site, but with poor pLDDT. **I)** GSx5 in magenta (GSGSGSGSGS) had a score (mean peptide from Simple Max method pLDDT) of 29.5. $(GGGGS)_2$ in orange (GGGGSGGGGS) had a score of 31.9. G_{10} in red (GGGGGGGGGG) had a score of 33. Lastly, **J)** A_{10} in cyan (AAAAAAAAAA) had a score of 41 and is the only negative control peptide to have an alpha-helical secondary structure (presumably due to the increased alpha helical propensity of alanine).

Supplemental Figure 6

PAbFold epitope detection is independent of position within target sequence.

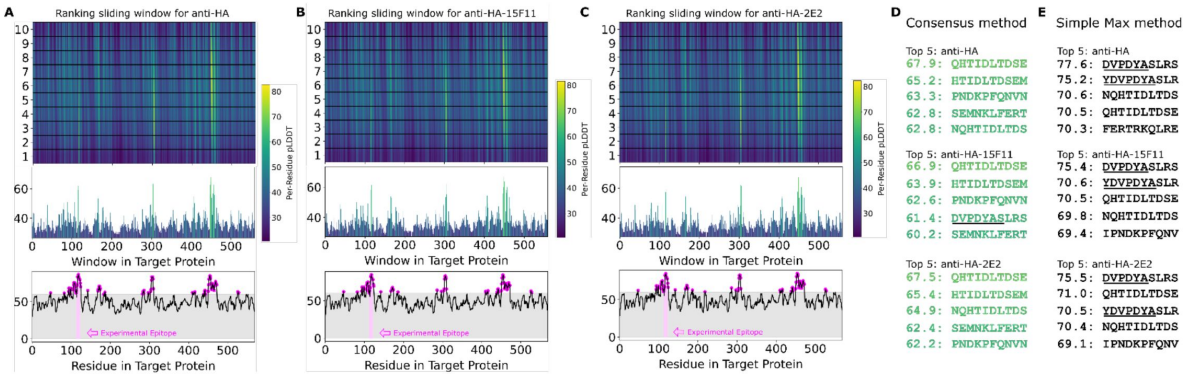
The Myc epitope (EQKLISEEDL) was added into the beginning, middle, or end of the 99-a.a. HIV protease sequence (Genbank Accession: NP_705926.1) prior to epitope scanning structure prediction. Positions of the Myc epitope sequence added to in the **A)** N-terminus **B)** middle and **C)** C-terminus of the HIV protease sequence. **D)** Highlights the ranked sequences recovered from each experiment in A, B, and C.

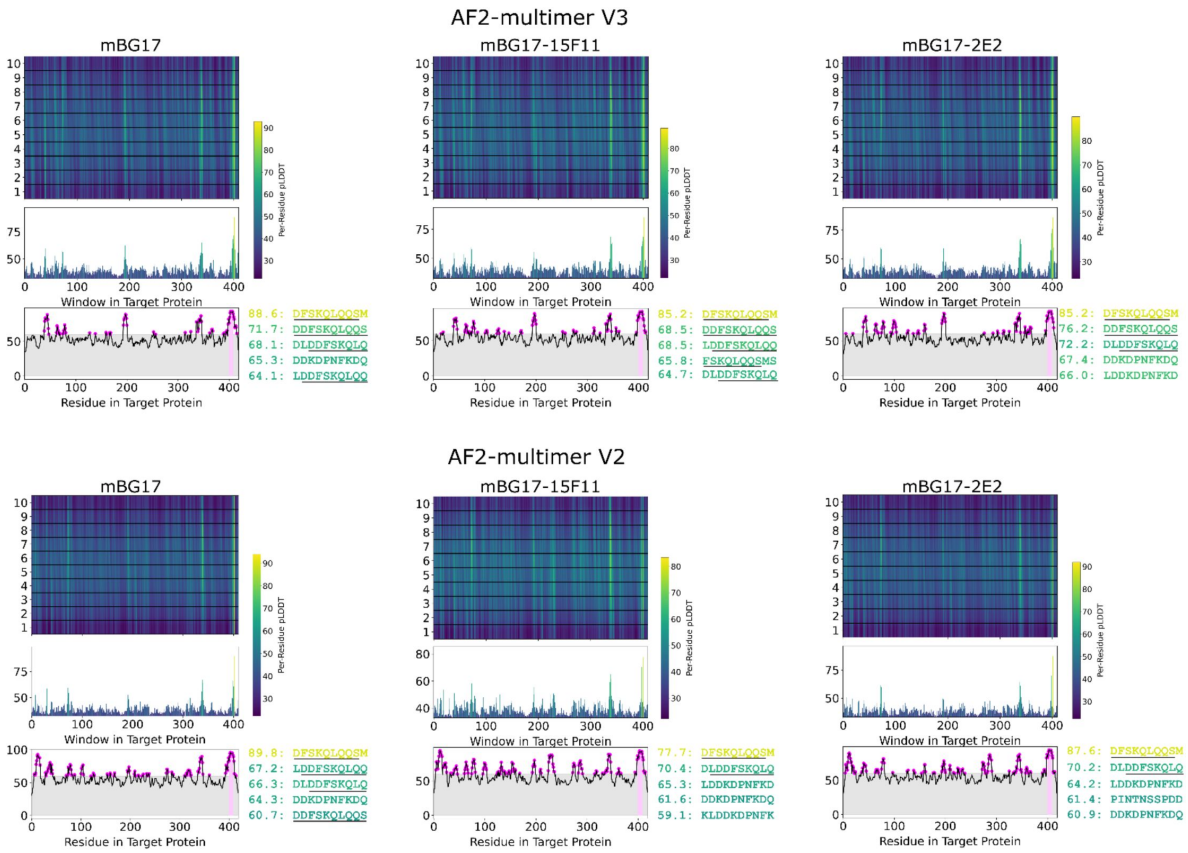


Supplemental Figure 7

Alphafold2 can accurately predict the HA linear epitope in different scFv backbones.

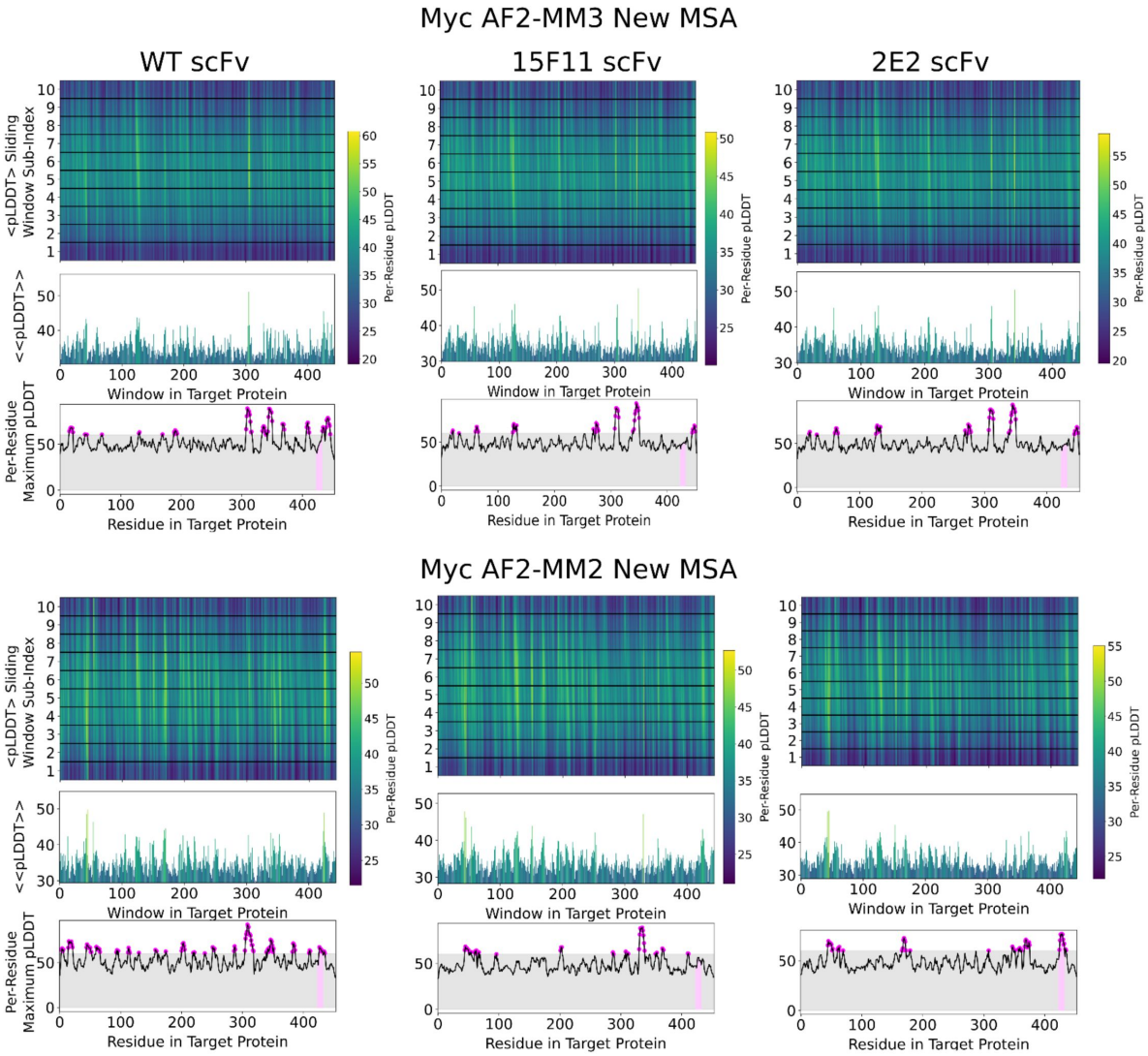
The anti-HA VH and VL antibody sequences were used to generate either **A)** wild-type scFv or CDR loop grafted onto the **B)** 15F11 or **C)** 2E2 antibody backbones. The Influenza A virus hemagglutinin protein sequence (Genbank AUT17530.1) was used as the target antigen and processed into 10 amino acid overlapping peptides with a 1 amino acid sliding window. The structures for each scFv:peptide pair were predicted with Alphafold2, and pLDDT values for each scFv:peptide pair are shown. **D)** The top-ranking epitope sequences via pLDDT scores are reported via the consensus method. Sequence underlining represents overlap with the known HA epitope (HA a.a. 114-125: YDVPDYASL). **E)** The top-ranking epitope sequences via pLDDT scores are reported via the simple max method.





Supplemental Figure 8

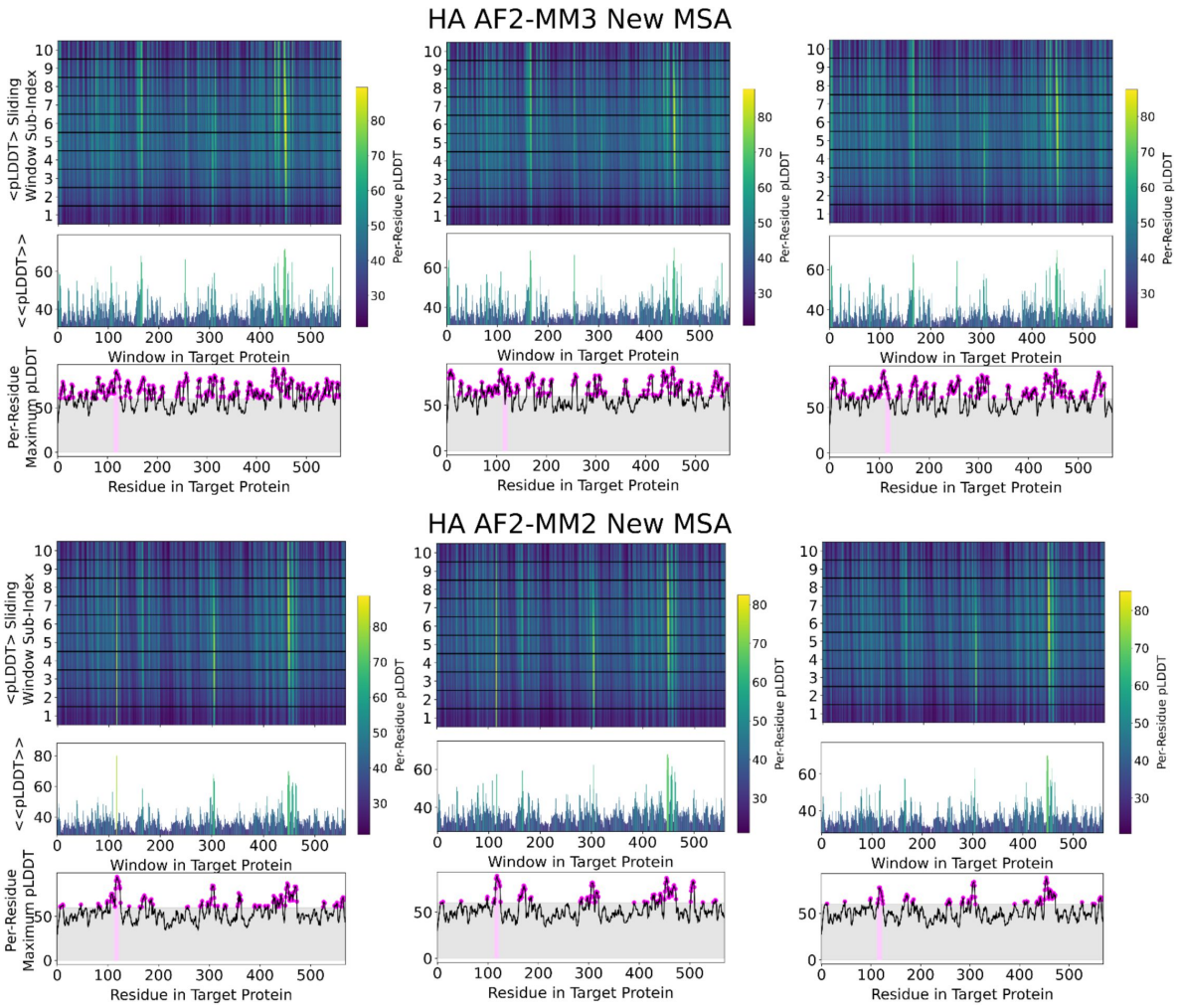
A comparison of Alphafold2 multimer version 3 and multimer version 2 applied to the mBG17 system. The experimental epitope, DDFSKQLQQS, is still easily identified with all three scFv backbones (wildtype, 15F11, and 2E2).



Supplemental Figure 9

Myc comparison of epitope identification accuracy, comparing model types.

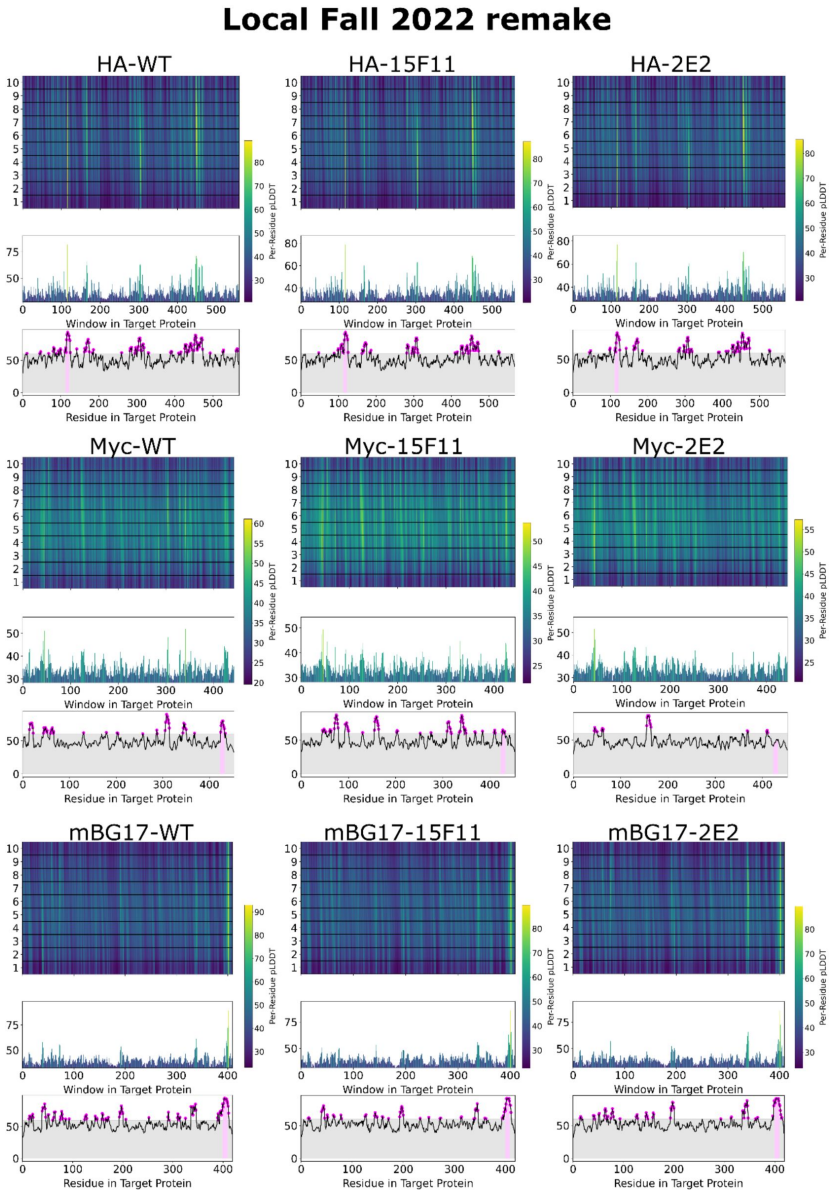
Performance variation with AlphaFold2 model (multiple versions 2 and 3) and MSA versions (most up to date version of the ColabFold MSA server uses UniRef30 (2302) and PDB100 (220517)) vs the old MSA server (when this data was initially generated, ColabFold MSA server used UniRef30 (2202) and PDB70 (220313)). The left column is the WT scFv, the middle column is the CDR loops spliced onto the 15F11 backbone, and the right column is the CDR loops spliced onto the 2E2 backbone. Performance was ablated when using MM3 and the new MSA, and significantly degraded when using MM2 with the new MSA. For AF2-MM2 Old MSA, see **Figure 2**.



Supplemental Figure 10

HA comparison of epitope identification accuracy, comparing model types.

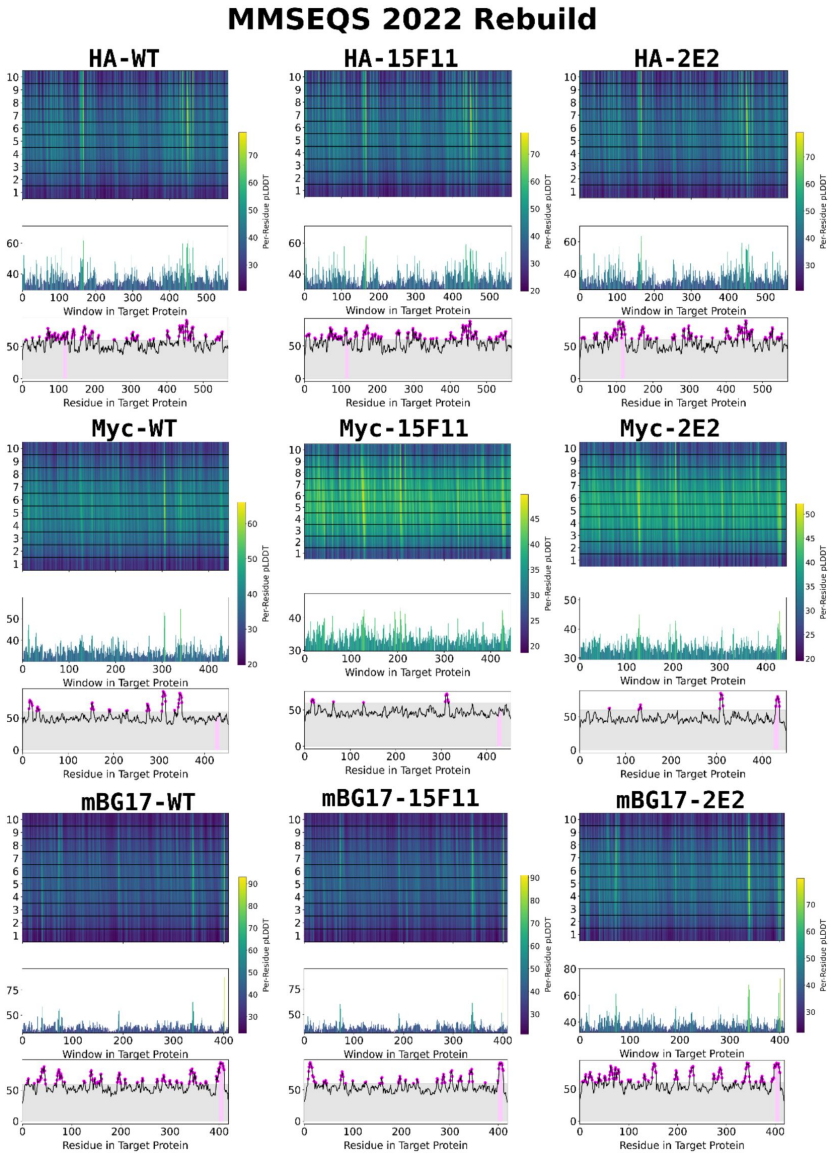
A comparison of the differing AlphaFold2 models with the Myc system (multimer version 3 and 2) along with a comparison of the new MSA (most up to date version of the ColabFold MSA server uses UniRef30 (2302) amd PDB100 (220517)) vs the old MSA server (when this data was initially generated, ColabFold MSA server used UniRef30 (2202) and PDB70 (220313)). The left column is the WT scFv, the middle column is the CDR loops spliced onto the 15F11 backbone, and the right column is the CDR loops spliced onto the 2E2 backbone. For AF2-MM2 Old MSA, see **Supplemental Figure 7**.



Supplemental Figure 11

Local remake of the databases used by the MMSEQS server.

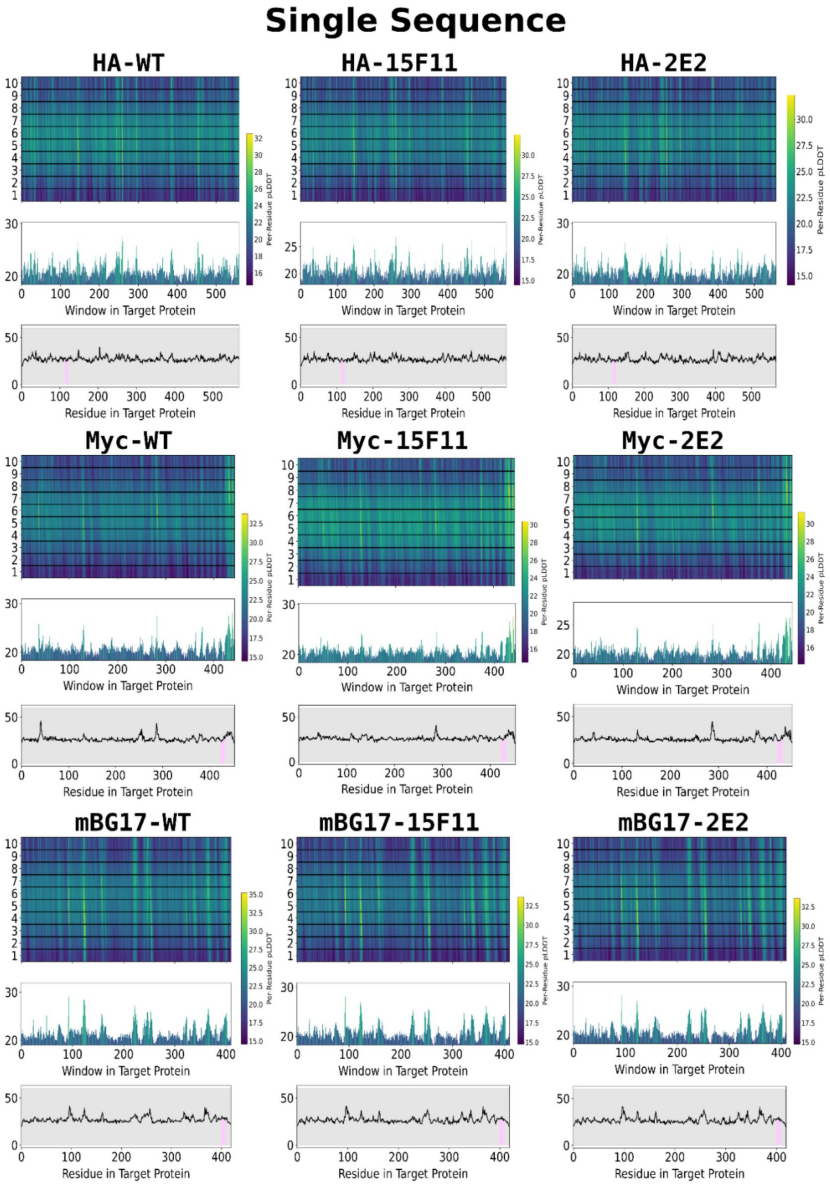
Databases were downloaded (UniRef30 (2202) and PDB70 (220313)) and were queried locally to produced MSA's for testing. These runs all were done with the multimer version 2 model of Alphafold 2. The left column is the WT scFv, the middle column is the CDR loops spliced onto the 15F11 backbone, and the right column is the CDR loops spliced onto the 2E2 backbone. The first row is the HA system, the second row is the Myc system, and the final row is the mBG17 system.



Supplemental Figure 12

Server remake of the MMSEQS databases.

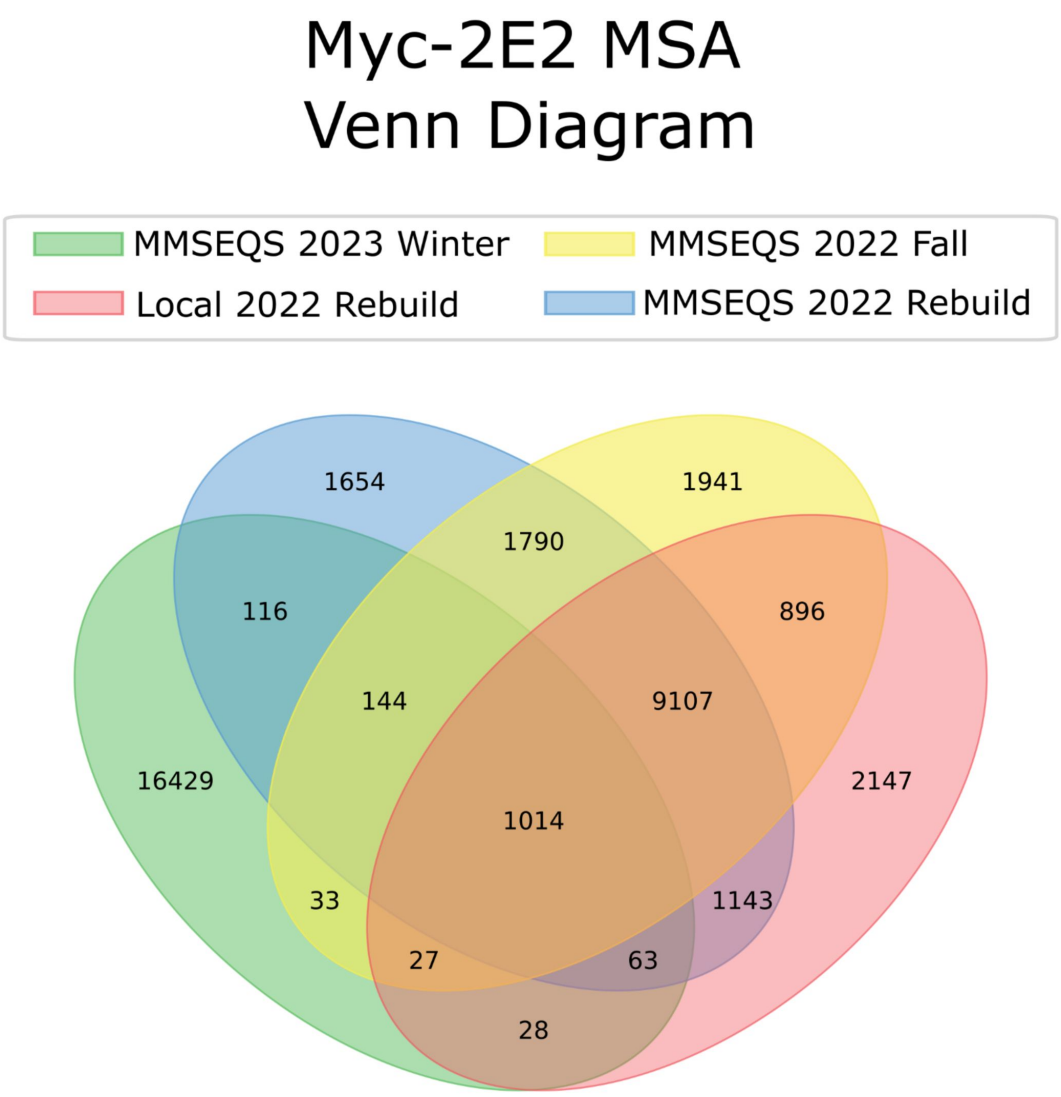
The databases were rebuilt by the MMSEQS team UniRef30 (2202) and PDB70 (220313)) on the Colabfold MSA server and were queried produced MSA's for testing. These runs all were done with the multimer version 2 model of Alphafold 2. The left column is the WT scFv, the middle column is the CDR loops spliced onto the 15F11 backbone, and the right column is the CDR loops spliced onto the 2E2 backbone. The first row is the HA system, the second row is the Myc system, and the final row is the mBG17 system.



Supplemental Figure 13

Single Sequence mode (no MSA's) of epitope prediction with AF2.

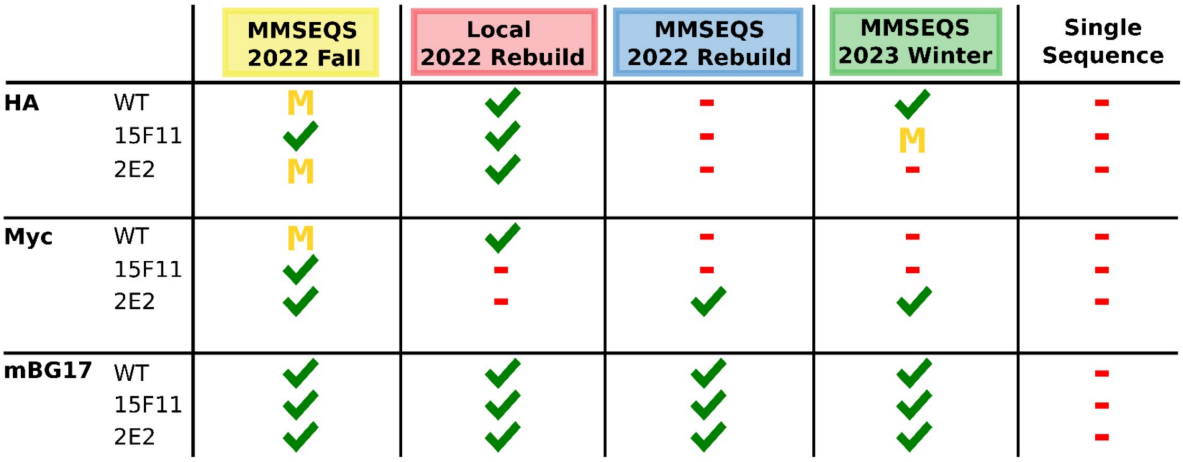
These runs all were done with the multimer version 2 model of Alphafold 2 in single sequence mode (i.e. no MSA was used) as a negative control, to highlight the importance of a quality MSA. The left column is the WT scFv, the middle column is the CDR loops spliced onto the 15F11 backbone, and the right column is the CDR loops spliced onto the 2E2 backbone. The first row is the HA system, the second row is the Myc system, and the final row is the mBG17 system.



Supplemental Figure 14

MSA overlap between the 4 generation methods.

Here we highlight the number of unique entries that are shared amongst all of the MSA methods, those being: **1)** using the databases right now via colabfold (PDB30 2302 and PDB100 230517) (green) **2)** the databases after they had been accessed via colabfold and cached for repeated use (UniRef30 (2202) and PDB70 (220313)) (yellow), **3)** downloading the databases locally (UniRef30 (2202) and PDB70 (220313)) and attempting to create the MSAs ourselves (red), and **4)** querying the databases after the MMSEQS team rebuilt them for our use via colabfold (UniRef30 (2202) and PDB70 (220 313)) (blue).



Supplemental Figure 15

Comparison of how well each MSA generation scheme accurately identified the experimentally derived epitope within the top 5 epitope sequences.

A green checkmark shows that it was found by both the consensus model and the top single model, a yellow "M" means the simple max method correctly identified the experimental epitope in the top 5 epitopes, and the red dash means both methods failed. The consensus model did not identify the epitope correctly when the simple max method failed to. The colored background behind the titles is the same color as **Supplemental Figure 14** to help guide the eye.

Acknowledgements

The authors would like to thank members of the Snow, Stasevich, and Geiss groups for helpful discussions at TagTeam meetings. This project was funded in part by NIH R01AI132668 (Geiss) and NIH R56AI155897 and R01AI168459 (Snow, Stasevich, Geiss). The authors would also like to thank Dr. Milot Mirdita for being our point of contact for the ColabFold and MMSEQS teams and assisting us with setting up databases and generating MSAs for our use.

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Editors

Reviewer #1 (Public Review):

Summary:

In this manuscript, "PAbFold: Linear Antibody Epitope Prediction using AlphaFold2", the authors generate a python wrapper for the screening of antibody-peptide interactions using AlphaFold, and test the performance of AlphaFold on 3 antibody-peptide complexes. In line with previous observations regarding the ability of AlphaFold to predict antibody structures and antigen binding, the results are mixed. While the authors are able to use AlphaFold to identify and experimentally validate a previously characterized broad binding epitope with impressive precision, they are unable to consistently identify the proper binding registers for their control [Myc-tag, HA-tag] peptides. Further, it appears that the reproducibility and generality of these results are low, with new versions of AlphaFold negatively impacting the predictive power. However, if this reproducibility issue is solved, and the test set is greatly increased, this manuscript could contribute strongly towards our ability to predict antibody-antigen interactions.

Strengths:

Due to the high significance, but difficulty, of the prediction of antibody-antigen interactions, any attempts to break down these predictions into more tractable problems should be applauded. The authors' approach of focusing on linear epitopes (peptides) is clever, reducing some of the complexities inherent to antibody binding. Further, the ability of AlphaFold to narrow down a previously broadly identified experimental epitope is impressive. The subsequent experimental validation of this more precisely identified epitope makes for a nice data point in the assessment of AlphaFold's ability to predict antibody-antigen interactions.

Weaknesses:

Without a larger set of test antibody-peptide interactions, it is unclear whether or not AlphaFold can precisely identify the binding register of a given antibody to a given peptide antigen. Even within the small test set of 3 antibody-peptide complexes, performance is variable and depends upon the scFv scaffold used for unclear reasons. Lastly, the apparent poor reproducibility is concerning, and it is not clear why the results should rely so strongly on which multi-sequence alignment (MSA) version is used, when neither the antibody CDR loops nor the peptide are likely to strongly rely on these MSAs for contact prediction.

Major Point-by-Point Comments:

(1) The central concern for this manuscript is the apparent lack of reproducibility. The way the authors discuss the issue (lines 523-554) it sounds as though they are unable to reproduce their initial results (which are reported in the main text), even when previous versions of AlphaFold2 are used. If this is the case, it does not seem that AlphaFold can be a reliable tool for predicting antibody-peptide interactions.

(2) Aside from the fundamental issue of reproducibility, the number of validating tests is insufficient to assess the ability of AlphaFold to predict antibody-peptide interactions. Given the authors' use of AlphaFold to identify antibody binding to a linear epitope within a whole protein (in the mBG17:SARS-Cov-2 nucleocapsid protein interaction), they should expand their test set well beyond Myc- and HA-tags using antibody-antigen interactions from existing large structural databases.

(3) As discussed in lines 358-361, the authors are unsure if their primary control tests (antibody binding to Myc-tag and HA-tag) are included in the training data. Lines 324-330 suggest that even if the peptides are not included in the AlphaFold training data because they contain fewer than 10 amino acids, the antibody structures may very well be included, with an obvious "void" that would be best filled by a peptide. The authors must confirm that their tests are not included in the AlphaFold training data, or re-run the analysis with these templates removed.

(4) The ability of AlphaFold to refine the linear epitope of antibody mBG17 is quite impressive and robust to the reproducibility issues the authors have run into. However, Figure 4 seems to suggest that the target epitope adopts an alpha-helical structure. This may be why the score is so high and the prediction is so robust. It would be very useful to see along with the pLDDT by residue plots a structure prediction by residue plot. This would help to see if the high confidence pLDDT is coming more from confidence in the docking of the peptide or confidence in the structure of the peptide.

(5) Related to the above comment, pLDDT is insufficient as a metric for assessing antibody-antigen interactions. There is a chance (as is nicely shown in Figure S3C) that AlphaFold can be confident and wrong. Here we see two orange-yellow dots (fairly high confidence) that place the peptide COM far from the true binding region. While running the recommended larger validation above, the authors should also include a peptide RMSD or COM distance metric, to show that the peptide identity is confident, and the peptide placement is roughly correct. These predictions are not nearly as valuable if AlphaFold is getting the right answer for the wrong reasons (i.e. high pLDDT but peptide binding to a non-CDR loop region). Eventual users of the software will likely want to make point mutations or perturb the binding regions identified by the structural predictions (as the authors do in Figure 4).

Comments on revisions:

I have read the author's responses and the revised manuscript. The authors did not sufficiently address my comments, nor the fundamental issue with the manuscript.

By the authors' own admission, many of the results presented in the current version of the manuscript cannot be reproduced without relying on locally saved MSAs. In other words, there is almost no evidence presented that this pipeline will predict antibody-antigen interactions using currently publicly available software. This manuscript is reduced to essentially a case study (N=1) in how one might go about making such predictions coupled with pretty good experimental evidence backing up this singular prediction.

https://doi.org/10.7554/eLife.98369.2.sa2

Reviewer #2 (Public Review):

Summary:

The authors showed the applicability and usefulness of a new AlphaFold2 pipeline called PabFold, which can predict linear antibody epitopes (B-cell epitopes) that can be helpful for the selection of reagents to be applied in competitive ELISA assay.

Strengths:

The authors showed the accuracy of the pipeline to identify correctly the binding epitope for three different antibody-antigen systems (Myc, HA, and Sars-Cov2 nucleocapsid protein). The design of scFvs from Fab of the three antibodies to speed up the analysis time is extremely interesting.

Weaknesses:

The article justifies correctly the findings and no great weaknesses are present. However, it could be useful for a broader audience to show in detail how pLDDT was calculated for both Simple-Max approach (per residue-pLDDT) and Consensus analysis (average pLDDT for each peptide), with associated equations.

Comments on revisions:

I have read the author's responses to my comments and the revised paper. They addressed the minor comments and concerns. However, I agree with Reviewer #1 that these findings cannot be reproduced without local MSAs and this is a major issue.

https://doi.org/10.7554/eLife.98369.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Reviewer 1 (Public Comments):***
>
> *(1) The central concern for this manuscript is the apparent lack of reproducibility. The way the authors discuss the issue (lines 523-554) it sounds as though they are unable to reproduce their initial results (which are reported in the main text), even when previous versions of AlphaFold2 are used. If this is the case, it does not seem that AlphaFold can be a reliable tool for predicting antibody-peptide interactions.*

The driving point behind the multiple sequence alignment (MSA) discussion was indeed to point out that AlphaFold2 (AF2) performance when predicting scFv:peptide complexes is highly dependent upon the MSA, but that is a function of MSA generation algorithm (MMseqs2, HHbiltz, jackhmmer, hhsearch, kalign, etc) and sequence databases, and less an intrinsic function of AF2. It is important to report MSA-dependent performance precisely because this results in changing capabilities with respect to peptide prediction.

Performance also significantly varies with the target peptide and scFv framework changes. By reporting the varying success rates (as a function of MSA, peptide target, and framework changes) we aim to help future researchers craft modified algorithms that can achieve increased reliability at protein-peptide binding predictions. Ultimately, tracking down how MSA generation details vary results (especially when the MSA's are hundreds long) is significantly outside the scope of this paper. Our goal for this paper was to show a general

method for identification of linear antibody epitopes using only sequence information, and future work by us or others should focus on optimization of the process.

> *(2) Aside from the fundamental issue of reproducibility, the number of validating tests is insufficient to assess the ability of AlphaFold to predict antibody-peptide interactions. Given the authors' use of AlphaFold to identify antibody binding to a linear epitope within a whole protein (in the mBG17:SARS-Cov-2 nucleocapsid protein interaction), they should expand their test set well beyond Myc- and HA-tags using antibody-antigen interactions from existing large structural databases.*

Performing the calculations at the scale that the reviewer is requesting is not feasible at this time. We showed in this manuscript that we were able to predict 3 of 3 epitopes, including one antigen and antibody pair that have not been deposited into the PDB with no homologs. While we feel that an N=3 is acceptable to introduce this method to the scientific community, we will consider adding more examples of success and failure in the future to optimize and refine the method as computational resources become available. Notably, future efforts that attempt high-throughput predictions of this class using existing databases should take particular care to avoid contamination.

> *(3) As discussed in lines 358-361, the authors are unsure if their primary control tests (antibody binding to Myc-tag and HA-tag) are included in the training data. Lines 324-330 suggest that even if the peptides are not included in the AlphaFold training data because they contain fewer than 10 amino acids, the antibody structures may very well be included, with an obvious "void" that would be best filled by a peptide. The authors must confirm that their tests are not included in the AlphaFold training data, or re-run the analysis with these templates removed.*

First, we address the simpler question of templates.

The reruns of AF2 with the local 2022 rebuild, the most reproducible method used with results most on par with the MMSEQS server in the Fall of 2022, were run without templates. This is because the MSA was generated locally; no templates were matched and generated locally. The only information passed then was the locally generated MSA, and the fasta sequence of the unchanging scFv and the dynamic epitope sequence. Because of how well this performed despite the absence of templates, we can confidently say the inclusion of the template flag is not significant with respect to how universally accurately PAbFold can identify the correct epitope.

Second, we can partially address the question of whether the AlphaFold models had access to models suitable, in theory, for “memorization” of pertinent structural details.

With respect to tracking the exact role and inclusion of specific PDB entries, the AF2 paper provides the following:

“Structures from the PDB were used for training and as templates (https://www.wwpdb.org/ftp/pdb-ftp-sites; for the associated sequence data and 40% sequence clustering see also https://ftp.wwpdb.org/pub/pdb/derived_data/ and https://cdn.rcsb.org/resources/sequence/clusters/bc-40.out). Training used a version of the PDB downloaded 28 August 2019, while the CASP14 template search used a version downloaded 14 May 2020. The template search also used the PDB70 database, downloaded 13 May 2020 (https://wwwuser.gwdg.de/~compbiol/data/hhsuite/databases/hhsuite_dbs/).”

Three of these links are dead. As such, it is difficult to definitively assess the role of any particular PDB entry with respect to AF2 training/testing, nor what impact homologous training structures given the very large number of immunoglobin structures in the training set. That said, we can summarize information for the potentially relevant PDB entries (l 2or9,

which is shown in Fig. 1 and 1frg), and believe it is most conservative to assume that each such entry was within the training set.

PDB entry 2or9 (released 2008): the anti-c-myc antibody 9E10 Fab fragment in complex with an 11-amino acid synthetic epitope: EQKLISEEDLN. This crystal structure is also noteworthy for featuring a binding mode where the peptide is pinned between two Fab. The apo structure (2orb) is also in the database but lacks the peptide and a resolved structure for CDR H3.

PDB entry 1a93 (released 1998): a c-Myc-Max leucine zipper structure, where the c-Myc epitope (in a 34-amino acid protein) adopts an alpha helical conformation completely different from the epitope captured in entry 2or9.

PDB entries 5xcs and 5xcu (released 2017): engineered Fv-clasps (scFv alternatives) in complex with the 9-amino acid synthetic HA epitope: YPYDVPDYA.

PDB entry 1frg (released 1994): anti-HA peptide Fab in complex with HA epitope subset Ace-DVPDYASL-NH2.

Since the 2or9 entry has our target epitope (10 aa) embedded within an 11aa sequence, we have revised this line in the manuscript:

The AlphaFold2 training set was reported to exclude chains of less than 10, which would eliminate the myc and HA epitope peptides. => The AlphaFold2 training set was reported to exclude chains of less than 10, which would eliminate the HA epitope peptide from potential training PDB entries such as 5xcs or 5xcu”

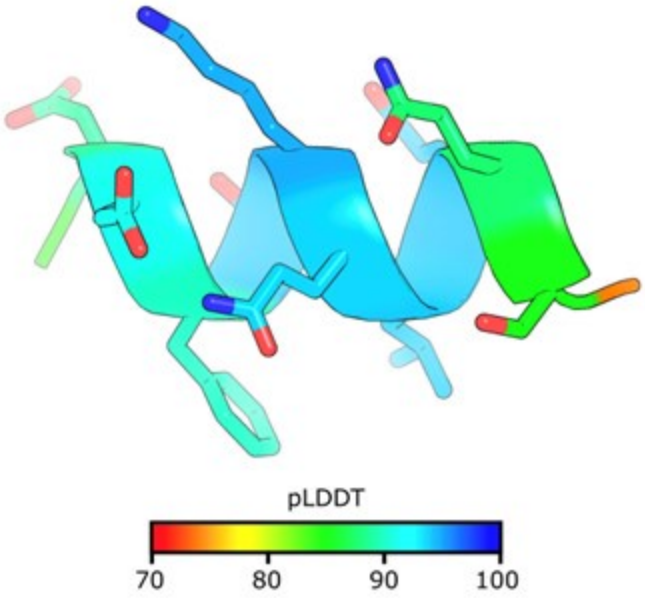
It is important to note that we obtained the best prediction performance for the scFv:peptide pair that had no pertinent PDB entries (mBG17). Specifically, doing a Protein Blast against the PDB using the mBG17 scFv revealed diverse homologs, but a maximum sequence identity of 89.8% for the heavy chain (to an unrelated antibody) and 93.8% for the light chain (to an unrelated antibody). Additionally, while it is possible that the AF2 models might have learned from the complex in pdb entry 2or9, Supplemental Figure 3 shows how often the peptide is “misplaced”, and the performance does not exceed the performance for mBG17.

> *(4) The ability of AlphaFold to refine the linear epitope of antibody mBG17 is quite impressive and robust to the reproducibility issues the authors have run into. However, Figure 4 seems to suggest that the target epitope adopts an alpha-helical structure. This may be why the score is so high and the prediction is so robust. It would be very useful to see along with the pLDDT by residue plots a structure prediction by residue plot. This would help to see if the high confidence pLDDT is coming more from confidence in the docking of the peptide or confidence in the structure of the peptide.*

The reviewer is correct that target mBG17 epitope adopts an alpha helical conformation, and we concur that this likely contributes to the more reliable structure prediction performance. When we predict the structure of the epitope alone without the mBG17 scFv, AF2 confidently predicts an alpha helix with an average pLDDT of 88.2 (ranging from 74.6 to 94.4).

Author response image 1.

The AF2 prediction for the mBG17 epitope by itself.



However, as one interesting point of comparison, a 10 a.a. poly-alanine peptide is also consistently folded into an alpha-helical coil by AF2. The A_{10} peptide is also predicted to bind among the traditional scFv CDR loops, but the pLDDT scores are very poor (Supplemental Figure 5J). We also observed the opposite case; when a peptide has a very unstructured region in the binding domain but is nonetheless still be placed confidently, as seen in Supplemental Figure 3 C&D. Therefore, while we suspect peptides with strong alpha helical propensity are more *likely* to be accurately predicted, the data suggests that that alpha helix adoption is neither necessary nor sufficient to reach a confident prediction.

> *(5) Related to the above comment, pLDDT is insufficient as a metric for assessing antibody antigen interactions. There is a chance (as is nicely shown in Figure S3C) that AlphaFold can be confident and wrong. Here we see two orange-yellow dots (fairly high confidence) that place the peptide COM far from the true binding region. While running the recommended larger validation above, the authors should also include a peptide RMSD or COM distance metric, to show that the peptide identity is confident, and the peptide placement is roughly correct. These predictions are not nearly as valuable if AlphaFold is getting the right answer for the wrong reasons (i.e. high pLDDT but peptide binding to a nonCDR loop region). Eventual users of the software will likely want to make point mutations or perturb the binding regions identified by the structural predictions (as the authors do in Figure 4).*

We agree with the reviewer that pLDDT is not a perfect metric, and we are following with great interest the evolving community discussion as to what metrics are most predictive of binding affinity (e.g. pAE, or pITM as a decent predictor for binding, but not affinity ranking). To our knowledge, there is not yet a consensus for the most predictive metrics for protein:protein binding nor protein:peptide binding. Intriguingly, since the antigen peptides are so small in our case, the pLDDT of the peptide residues should be mostly reporting on the confidence of the distances to neighboring protein residues.

As to the suggestion for a RMSD or COM distance metric, we agree that these are useful -with the caveat that these require a reference structure. The goal of our method is to quickly narrow down candidate linear epitopes and thereby guide experimentalists to more

efficiently determine the actual binding sequence of an antibody-antigen sequence. Presumably this would not be necessary if a reference structure were known.

It may also be possible to invent a method to filter unlikely binding modes that is specific to antibodies and peptide epitopes that does not require a known reference structure, but this would be an interesting problem for subsequent study.

> ***Reviewer 1 (Recommendations for the Authors):***
>
> *(1) "Linear epitope" should be more precisely defined in the text. It isn't clear whether the authors hope that they can use AlphaFold to predict where on a given protein antigen an antibody will bind, or which antigenic peptide the antibody will bind to. The authors discuss both problems, and there is an important distinction between the two. If the authors are only concerned with isolated antigenic peptides, rather than linear epitopes in their full length structural contexts, they should be more precise in the introduction and discussion.*

We thank the reviewer for the prompt towards higher precision. We are using the short contiguous antigen definition of "linear epitope" that depends on secondary rather than tertiary structure. The linear epitopes this paper considers are short "peptides" that form secondary structure independent of their structure in the complete folded antigen protein. We have clarified our definition of "linear epitope" in the text (lines 64-66).

> *(2) Line 101: "Not all portions of the antibody are critical". First, this is not consistent with the literature, particularly where computational biology is concerned.*
>
> *See https://pubs.acs.org/doi/10.1021/acs.jctc.7b00080 . Second, while I largely agree with what I think the authors are trying to say (that we can largely reduce the problem to the CDR loops), this is inconsistent with what the authors later find, which is that inexplicably the VH/VL scaffold used alters results strongly.*

We have adopted verbiage that should be less provocative: "Fortunately, with respect to epitope specificity, antibody constant domains are less critical than the CDR loops and the remainder of the variable domain framework regions."

> *(3) Related to the above comment, do the authors have any idea why epitope prediction performance improved for the chimeric scFvs? Is this due to some stochasticity in AlphaFold? Or is there something systematic? Expanding the test dataset would again help answer this question.*

We agree that future study with a larger test set could help address this intriguing result, for which we currently lack a conclusive explanation. Part of our motivation for this publication was to bring to light this unexpected result. Notably, these framework differences are not only implicated as a factor in driving AF2 performance, but also changing experimental intracellular performance as reported by our group (DOI: 10.1038/s41467-019-10846-1). We can generate a variety of hypotheses for this phenomenon. Just as MSA sub-sampling has been a popular approach to drive AF2 to sample alternative conformations, sequence recombination may be a generically effective way to generate usefully different binding predictions. However, it is difficult to discriminate between recombination inducing subtle structural tweaks that increase protein intracellular fitness and binding, from recombination causing changes to the MSA that affect the likelihood of sampling a good epitope binding conformation. It is also possible that the chimeras are more deftly predicted by AF2 due to differences in sequence representation during the training of the AF2 models (e.g. more exposure to models containing 15F11 or 2E2 structures). We attempted to deconvolute MSA

differences by using single-sequence mode (Supplementary Figure 13) but this ablated performance.

> *(4) Figure 2: The reported consensus pLDDT scores are actually quite low here, suggesting low confidence in the result. This is in strong contrast to the reported consensus scores for mBG17. Again, a larger test dataset would help set a quantitative cutoff for where to draw the line for "trustworthy" AlphaFold predictions in antibody-peptide binding applications.*

We agree that a larger dataset will be useful to begin to establish metrics and thresholds and will contribute to the aforementioned community discussion about reliable predictors of binding. Our current focus is not structure prediction per se. In the current work we are more focused on *relative* binding likelihood and increasing the efficiency of experimental epitope verification by flagging the most likely linear epitopes. Thus, while the pLDDT scores are low for Myc in Figure 2, it is remarkable (and worth reporting) that there is still useful signal in the relative variation in pLDDT. The utility of the signal variation is evident in the ability to short-list correct lead peptides via the two methods we demonstrate (consensus and per-residue max).

> *(5) Figure 4: if the authors are going to draw conclusions from the actual structure predictions of AlphaFold (not just the pLDDT scores), the side-chain accuracy placement should be assessed in the test dataset (RMSD or COM distance).*

We agree with the reviewer that side-chain placement accuracy is important when evaluating the accuracy of AF2 structure predictions. However, here our focus was relative binding likelihood rather than structure prediction. The one case where we attempted to draw conclusions from the structure prediction was in the context of mBG17, where there is not yet an experimental reference structure. Absolutely, if we were to obtain a crystal structure for that complex, we would assess side-chain placement accuracy.

> *(6) Lines 493-508: I am not sure that this assessment for why AlphaFold has difficulty with antibody-antigen interactions is correct. If the authors' interpretation is correct (larger complicated structures are more challenging to move) then AlphaFold-Multimer (https://www.biorxiv.org/content/10.1101/2021.10.04.463034v2.full) wouldn't perform as well as it does. Instead, the issue is likely due to the incredibly high diversity in antibody CDR loops, which reduces the ability of the AlphaFold MSA step (which the authors show is quite critical to predictions: Figure S13) to inform structure prediction. This, coupled with the importance of side chain placement in antibody and TCR interactions, which is notoriously difficult (https://elifesciences.org/articles/90681), are likely the largest source of uncertainty in antibody-antigen interaction prediction.*

We agree with the reviewer that CDR loop diversity (and associated side chain placement challenges) are a major barrier to successfully predict antibody-antigen complexes. Presumably this is true for both peptide antigens and protein antigens. Indeed, the authors of AlphaFold-multimer admit that the updated model struggles with antibody-antigen complexes, saying “As a limitation, we observe anecdotally that AlphaFold-Multimer is generally not able to predict binding of antibodies and this remains an area for future work.” The point about how loop diversity could reduce MSA quality is well taken. We have included the following thanks to the guidance of the reviewer when discussing MSA sensitivity is discussed later on in lines 570-572.:

“These challenges are presumably compounded by the incredible diversity of the CDR loops in antibodies which could decrease the useful signal from the MSA as well as drive inconsistent MSA-dependent performance”.

With respect to lines 493-508, we have also rephrased a key sentence to try to better explain that we are comparing the often-good recognition performance for short epitopes to the never-good performance when those epitopes are embedded within larger sequences. Instead of saying, “In contrast, a larger and complicated structure may be more challenging to move during the AlphaFold2 structure prediction or recycle steps.” we now say in lines 520-522 , “In contrast, embedding the epitope within a larger and more complicated structure appears to degrade the ability of AlphaFold2 to sample a comparable bound structure within the allotted recycle steps.”

> *(7) Related to major comment 1: Are AlphaFold predictions deterministic? That is, if you run the same peptide through the PAbFold pipeline 20 times, will you get the same pLDDT score 20 times? The lack of reproducibility may be in part due to stochasticity in AlphaFold, which the authors could actually leverage to provide more consistent results.*

This is a good question that we addressed while dissecting the variable performance. When the random seed is fixed, AF2 returns the same prediction every time. After running this 10 times with a fixed seed, the mBG17 epitope was predicted with an average pLDDT of 88.94, with a standard deviation of 1.4×10^{-14}. In contrast, when no seed is specified, AF2 did not return an *identical* result. However, the results were still remarkably consistent. Running the mBG17 epitope prediction 10 times with a different seed gave an average pLDDT of 89.24, with a standard deviation of 0.49.

> *(8) Related to major comment 2: The authors could use, for example, this previous survey of 1833 antibody-antigen interactions (https://www.sciencedirect.com/science/article/pii/S2001037023004725) the authors could likely pull out multiple linear epitopes to test AlphaFold's performance on antibody peptide interactions. A large number of tests are necessary for validation.*

We thank the reviewer for this report of antibody-antigen interactions and will use it as a source of complexes in a future expanded study. Given the quantity and complexity of the data that we are already providing, as well as logistical challenges for compute and personnel the reviewer is asking for, we must defer this expansion to future work.

> *(9) Related to major comment 3: Apologies if this is too informal for a review, but this Issue on the AlphaFold GitHub may be useful: https://github.com/googledeepmind/alphafold/issues/416 .*

We thank the reviewer for the suggestion – per our response above we have indeed run predictions with no templates. Since we are using local AlphaFold2 calculations with localcolabfold, the use or non-use of templates is fairly simple: including a “—templates” flag or not.

> *(10) Related to major comment 4: I am not sure if AlphaFold outputs by-residue secondary structure prediction by default, but I know that Phyre2 does http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index .*

To our knowledge, AF2 does not predict secondary structure independent of the predicted tertiary structure. When we need to analyze the secondary structure we typically use the program DSSP from the tertiary structure.

> *(11) The documentation for this software is incomplete. The GitHub ReadMe should include complete guidelines for users with details of expected outputs, along with a thorough step-by-step walkthrough for use.*

We thank the reviewer for pointing this out, but we feel that the level of detail we provide in the GitHub is sufficient for users to utilize the method described.

> *Stylistic comments:*
>
> *(1) I do not think that the heatmaps (as in 1C, top) add much information for the reader. They are largely uniform across the y-axis (to my eyes), and the information is better conveyed by the bar and line graphs (as in 1C, middle and bottom panels).*

We thank the reviewer for this feedback but elect to leave it in on the premise of more data presented is (usually) better. Including the y-axis reveals common patterns such as the lower confidence of the peptide termini, as well as the lack of some patterns that might have occurred. For example, if a subset of five contiguous residues was necessary and sufficient for local high confidence this could be visually apparent as a "staircase" in the heat map.

> *(2) A discussion of some of the shortcomings of other prediction-based software (lines 7177) might be useful. Why are these tools less well-equipped than AlphaFold for this problem? And if they have tried to predict antibody-antigen interactions, why have they failed?*

We agree with the reviewer that a broader review of multiple methods would be interesting and useful. One challenge is that the suite of available methods is evolving rapidly, though only a subset work for multimeric systems. Some detail on deficiencies of other approaches was provided in lines 71-77 originally, although we did not go into exhaustive detail since we wanted to focus on AF2. We view using AF2 in this manner is novel and that providing additional options predict antibody epitopes will be of interest to the scientific community. We also chose AF2 because we have ample experience with it and is a software that many in the scientific community are already using and comfortable with. Additionally, AF2 provided us with a quantification parameter (pLDDT) to assess the peptides' binding abilities. We think a future study that compares the ability of multiple emerging tools for scFv:peptide prediction will be quite interesting.

> *(3) Similar to the above comment, more discussion focused on why AlphaFold2 fails for antibodies (lines 126-128) might be useful for readers.*

We thank the reviewer for the suggestion. The following line has been added shortly after lines 135-137:

"Another reason for selecting AF2 is to attempt to quantify its abilities the compare simple linear epitopes, since the team behind AF-multimer reported that conformational antibody complexes were difficult to predict accurately (14)."

Per earlier responses, we also added text that flags one particular possible reason for the general difficulty of predicting antibody-antigen complexes (the diversity of the CDR loops and associated MSA challenges).

> *(4) The first two paragraphs of the results section (lines 226-254) could likely be moved to the Methods. Additionally, details of how the scores are calculated, not just how the commands are run in python, would be useful.*

Per the reviewer suggestion, we moved this section to the end of the Methods section. Also, to aid in the reader's digestion of the analysis, the following text has been added to the Results section (lines 256-264):

"Both the 'Simple Max' and 'Consensus' methods were calculated first by parsing every pLDDT score received by every residue in the antigen sequence sliding window output structures. From the resulting data structure, the Simple Max method simply finds the maximum pLDDT value ever seen for a single residue (across all sliding windows and AF2 models). For the Consensus method, per-residue pLDDT was first averaged across the 5 AF2 models. These averages are reported in the heatmap view, and further averaged per sliding window for the bar chart below.

In principle, the strategy behind the Consensus method is to take into account agreement across the 5 AF2 models and provide insight into the confidence of entire epitopes (whole sliding windows of n=10 default) instead of disconnected, per-residue pLDDT maxima."

> *(5) Figure 1 would be more useful if you could differentiate specifically how the Consensus and Simple Max scoring is different. Providing examples for how and why the top 5 peptide hits can change (quite significantly) using both methods would greatly help readers understand what is going on.*

Per the reviewer suggestion, we have added text to discuss the variable hit selection that results from the two scoring metrics. The new text (lines 264-271) adds onto the added text block immediately above:

"Having two scoring metrics is useful because the selection of predicted hits can differ. As shown in Figure 2, part of the Myc epitope makes it into the top 5 peptides when selection is based on summing per-residue maximum pLDDT (despite there being no requirement that these values originate in the same physical prediction). In contrast, a Consensus method score more directly reports on a specific sliding window, and the strength of the highest confidence peptides is more directly revealed with superior signal to noise as shown in Figure 3. Variability in the ranking of top hits between the two methods arises from the fundamental difference in strategy (peptide-centric or residue-centric scoring) as well as close competition between the raw AF2 confidence in the known peptide and competing decoy sequences."

> *(6) Hopefully the reproducibility issue is alleviated, but if not the discussion of it (lines 523554) should be moved to the supplement or an appendix.*

The ability of the original AF2 model to predict protein-protein complexes was an emergent behavior, and then an explicit training goal for AF2.multimer. In this vein, the ability to predict scFv:peptide complexes is also an emergent capability of these models. It is our hope that by highlighting this capacity, as well as the high level of sensitivity, that this capability will be enhanced and not degraded in future models/algorithms (both general and specialized). In this regard, with an eye towards progress, we think it is actually important to put this issue in the scientific foreground rather than the background. When it comes to improving machine learning methods negative results are also exceedingly important.

> ***Reviewer 2 (Recommendations for the Author):***
>
> *- Line 113, page 3 - the structures of the novel scFv chimeras can be rapidly and confidently be predicted by AlphaFold2 to the structures of the novel scFv chimeras can be rapidly and confidently predicted by AlphaFold2.*

The superfluous "be" was removed from the text.

> *- Line 276 and 278 page 9 - peptide sequences QKLSEEDLL and EQKLSEEDL in the text are different from the sequences reported in Figures 1 and 2 (QKLISEEDLL and EQKLISEEDL). Please check throughout the manuscript and also in the Figure caption (as in Figure 2).*

These changes were made throughout the text.

> *- I would include how you calculate the pLDDT score for both Simple Max approach and Consensus analysis.*

Good suggestion, this should be covered via the additions noted above.

https://doi.org/10.7554/eLife.98369.2.sa0