De novo Sequencing and Kinetic Characterization of Alpaca Polyclonal Antibodies

Rosalin Dubois[1] Tess Nunez[1] Chelsea Reitzel[1] Amber Couzens[1] Jin Duan[1] Lin Wu[1] Qixin Liu[1] Thierry Le Bihan[1] Marko Jović[1] Bin Ma[1]

[1]Rapid Novor, Inc., Kitchener, ON, Canada

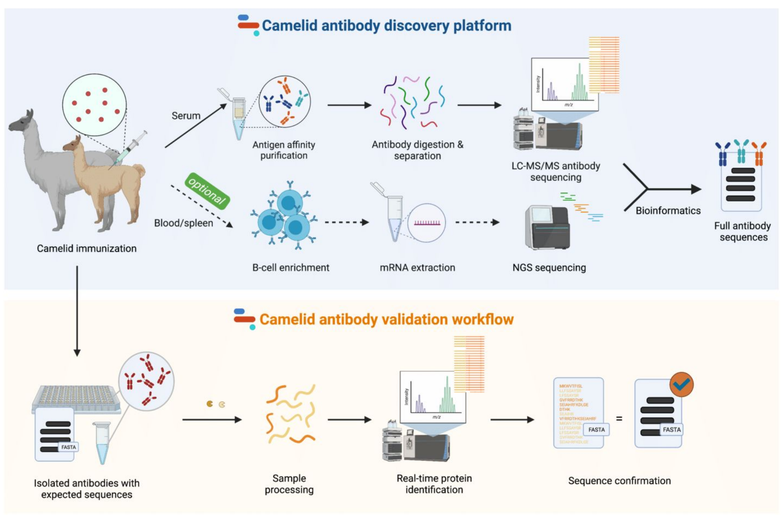
BACKGROUND

A subclass of Camelid antibodies, devoid of light chains, are a structurally unique family of immunoglobulins (IgGs) that carry a significant potential for biologics development given their small size, stability and high affinity. In particular, recombinant VHH regions of camelid IgGs (also referred to as nanobodies) have been at the focus of recent antibody engineering efforts, given their excellent tissue penetration compared to full size IgGs and monovalency. Although those properties make them easy to manufacture, the biggest challenge in realizing the full potential of nanobodies is the initial elucidation of the protein sequence. Standard sequencing approach involves next generation sequencing (NGS) from the circulating peripheral blood lymphocytes, or lymph or spleen, followed by generation of a phage display library used to perform affinity maturation *in vitro*.

SIGNIFICANCE

In this study, we are using a novel sequencing approach, by sequencing alpaca IgGs directly from serum proteins *de novo*, thus eliminating the need for genomic analysis.
This is the **first successful study to sequence alpaca IgG proteome *de novo***, with a technology that directly sequenced IgGs from the immune repertoire, thus identifying more physiologically relevant, high affinity binders while not being limited by the availability of B-cells in circulation.

POLYCLONAL SEQUENCING METHODOLOGY

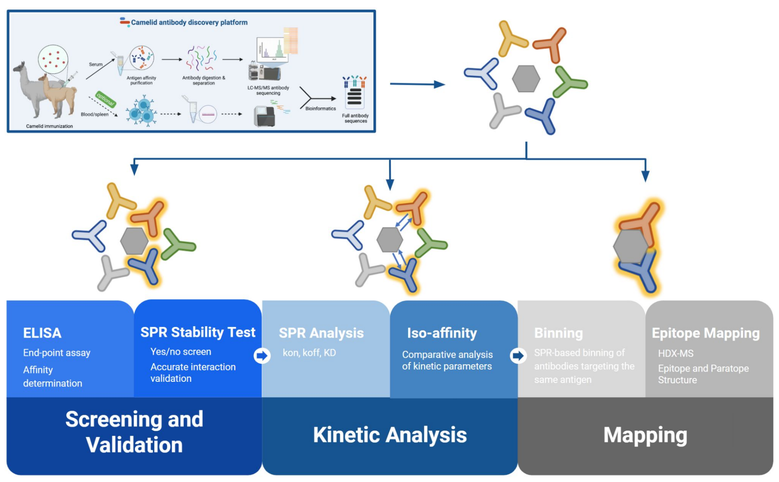


In this study we used a Next Generation Protein Sequencing (NGPS) platform for the discovery and validation of camelid antibodies and nanobodies. Serum was collected from an alpaca immunized with a rabbit polyclonal antigen. Next, serum samples were affinity purified, fractionated and digested. Sequencing of the alpaca polyclonal samples was performed using mass spectrometry. In particular, Liquid Chromatography tandem MS (LC-MS/MS) was performed on an Orbitrap Fusion Tribid instrument.

Using our established REpAb sequencing platform and a machine learning-based bioinformatic analysis we were able to successfully compile full amino acid sequences of individual alpaca IgG clones found in the original polyclonal mixture. Further validation of alpaca monoclonal sequences was performed against known FASTA sequences.

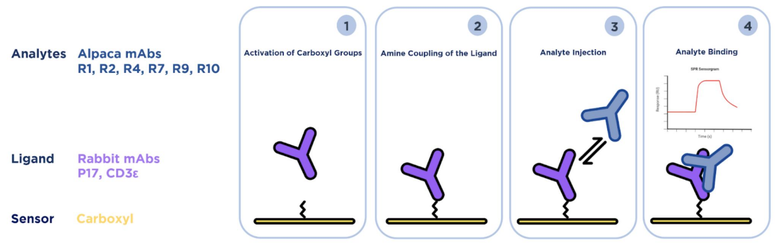
A total of 10 sequences were identified, from that pool of sequenced alpaca antibodies, six monoclonal IgGs representative were selected for synthesis of recombinant monoclonal antibodies and further development.

ALPACA MONOCLONAL ANTIBODY LEAD CHARACTERIZATION

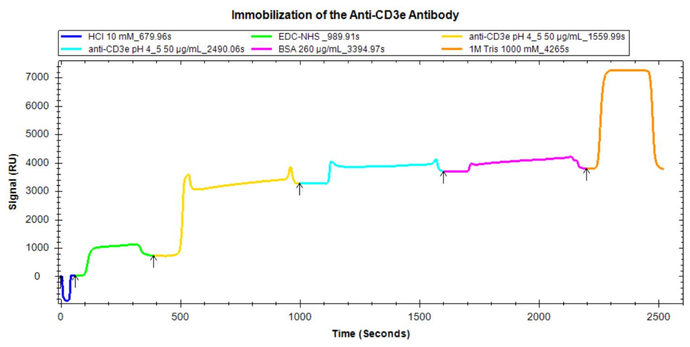


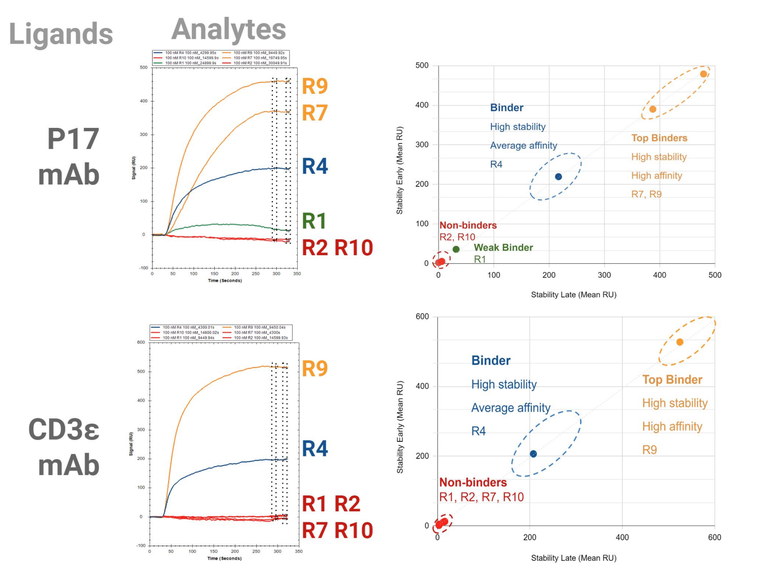
SCREENING AND VALIDATION

Stability screening (Yes/No binding screen) was performed by surface plasmon resonance (SPR) along with an ELISA affinity screen. Two rabbit monoclonal targets (P17 and CD3e mAbs) were chosen based on the original antigen pool used for alpaca immunization.

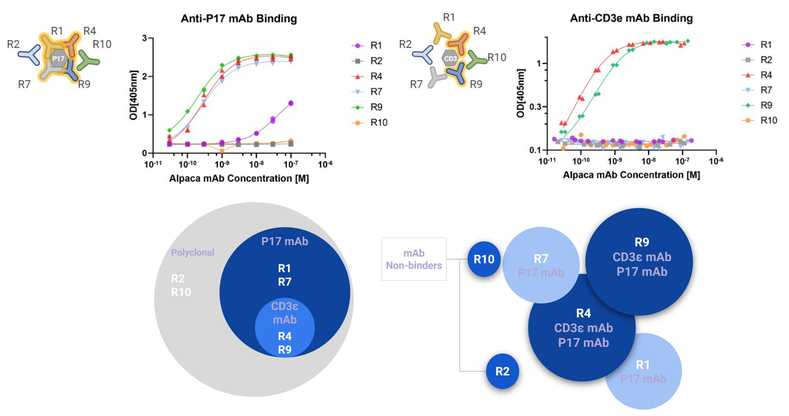


Top six alpaca monoclonal antibodies displayed a range of affinities towards the two targets with two clones showing displaying selectivity towards P17.





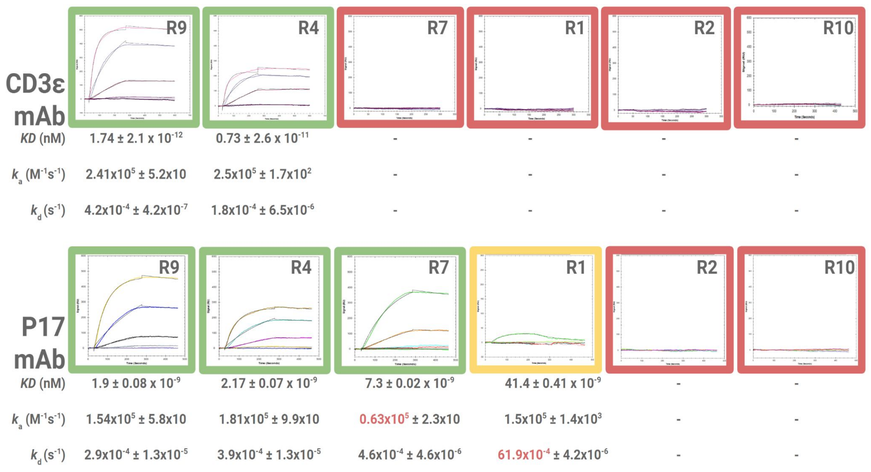
Orthogonal study by ELISA validated those results and confirmed the affinity ranking obtained through the SPR screen.



KINETIC PROFILING OF TOP CANDIDATES

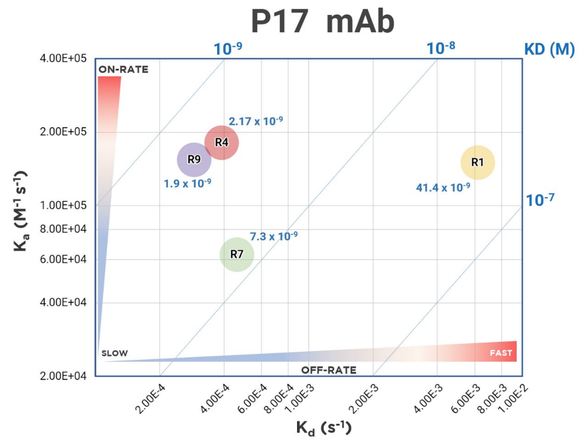
Four lead alpaca monoclonal candidates identified by stability screening and validated by ELISA were further characterized by SPR to determine their full kinetic profiles (k_{on}, k_{off} and *KD*).

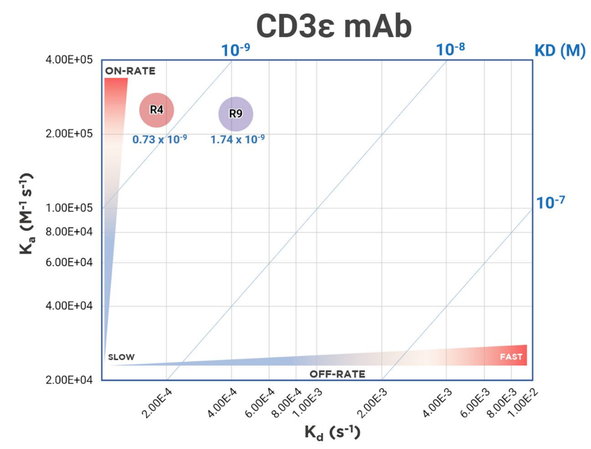
Of the four binders, each exhibited a unique binding profile with a potential for further downstream antibody development in a variety of applications. Key defining characteristics of the set are the slow on-rate of the R7 clone ($0.63x10^5$ $M^{-1}s^{-1}$) and the fast off-rate of the R1 clone ($61.9x10^{-4}$ s^{-1}).



ISO-AFFINITY ANALYSIS

Comparative affinity analysis was performed to dissect individual contributions of each kinetic parameter for each alpaca mAb. In particular, the association rate (y-axis) was plotted against the dissociation rate (x-axis) to identify individual trends contributing to each of the KD measurements.





KINETIC CHARACTERIZATION INSIGHTS

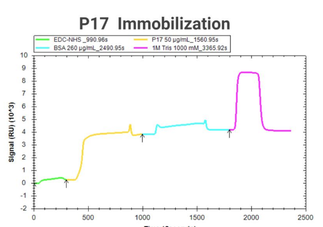
R4 and R9 clones were found to **have similar kinetic profiles with high affinity towards both P17 and CD3e** mAb targets, with a slight preference for CD3e mAb.

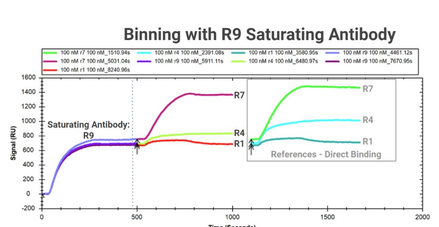
Conversely, R1 was found to have ~20x lower affinity despite having a very similar on-rate to R4 and R9. **Primary driver of the lower affinity of R1 is the fast off-rate, that is an order of magnitude higher than that of R4, R7 and R9**.

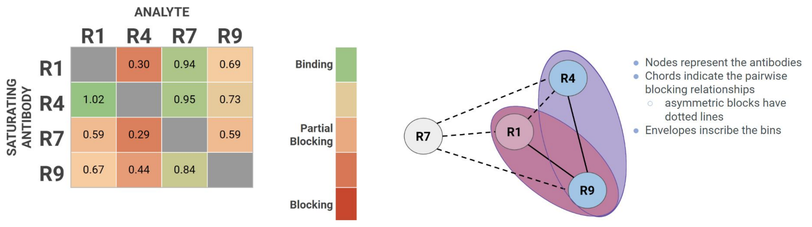
Finally, the **weaker affinity of R7 towards P17 is primarily due to the slower on-rate** in comparison to all the other alpaca mAb clones.

EPITOPE BINNING

The epitope diversity on P17 was evaluated for R1, R4, R7 and R9 using a tandem binning approach. Each of the clones was injected at a saturating 100 nM concentration and used either as a saturating antibody or a binding antibody in a 4x4 binning format.







SUMMARY

Sequencing of the alpaca IgG proteome *de novo* is capable of identifying the physiologically relevant, high affinity binders while not being limited by the availability of B-cells in circulation.

Synergistic approach between machine-learning driven proteomics and SPR makes it possible to accelerate the camelid nanobody discovery workflow by minimizing the need to produce the entire collection of potential candidate antibodies.