

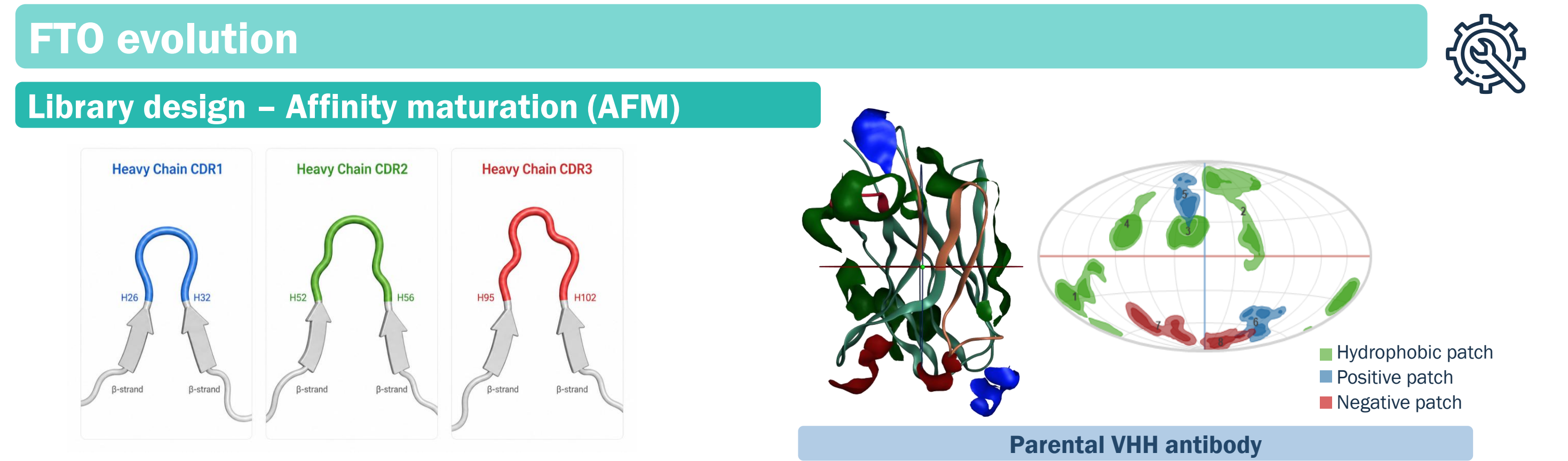
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# Balancing Innovation and Developability: A Dual Strategy for Delivering Clinical-Grade VHH Antibodies

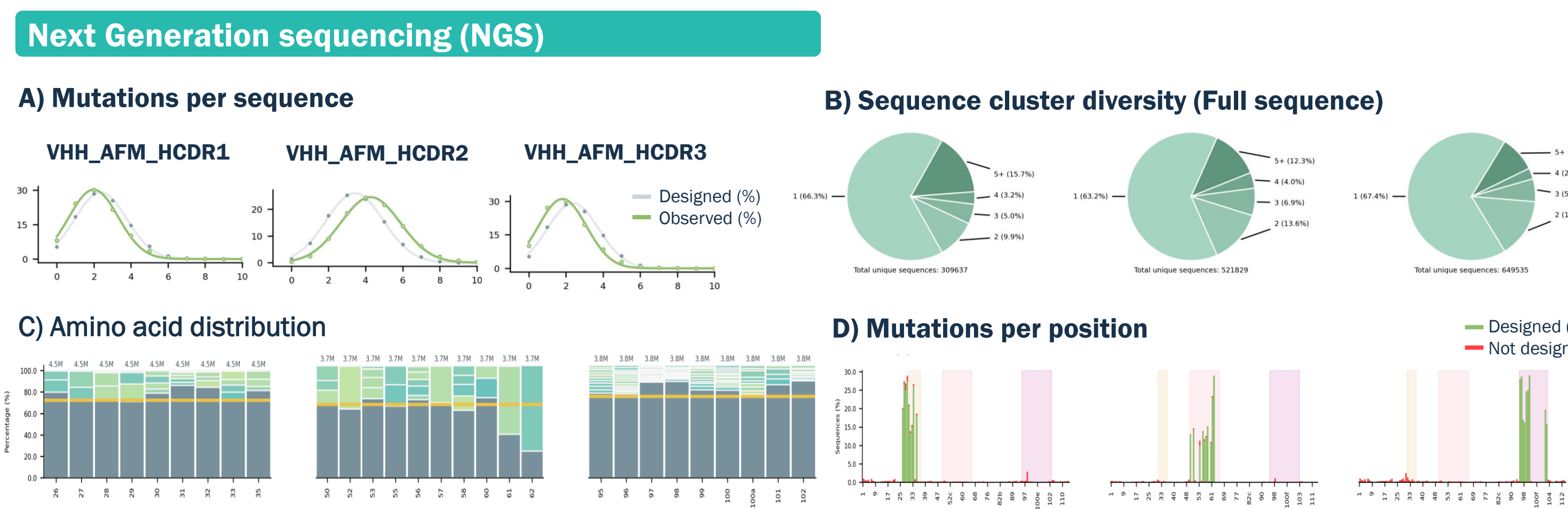
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The discovery of therapeutic antibodies requires a careful balance between molecular innovation and downstream developability to ensure successful clinical translation. Single-domain antibodies (VHs) offer unique advantages, including small size, high stability, and access to challenging epitopes, but their optimization must consider both functional performance and developability risks early in the pipeline. To address these challenges, integrated discovery strategies that combine rational engineering with immune-derived diversity are increasingly valuable. Engineering-based approaches enable precise control over sequence space and can support Freedom-to-Operate (FTO) objectives, while in vivo immunization campaigns leverage

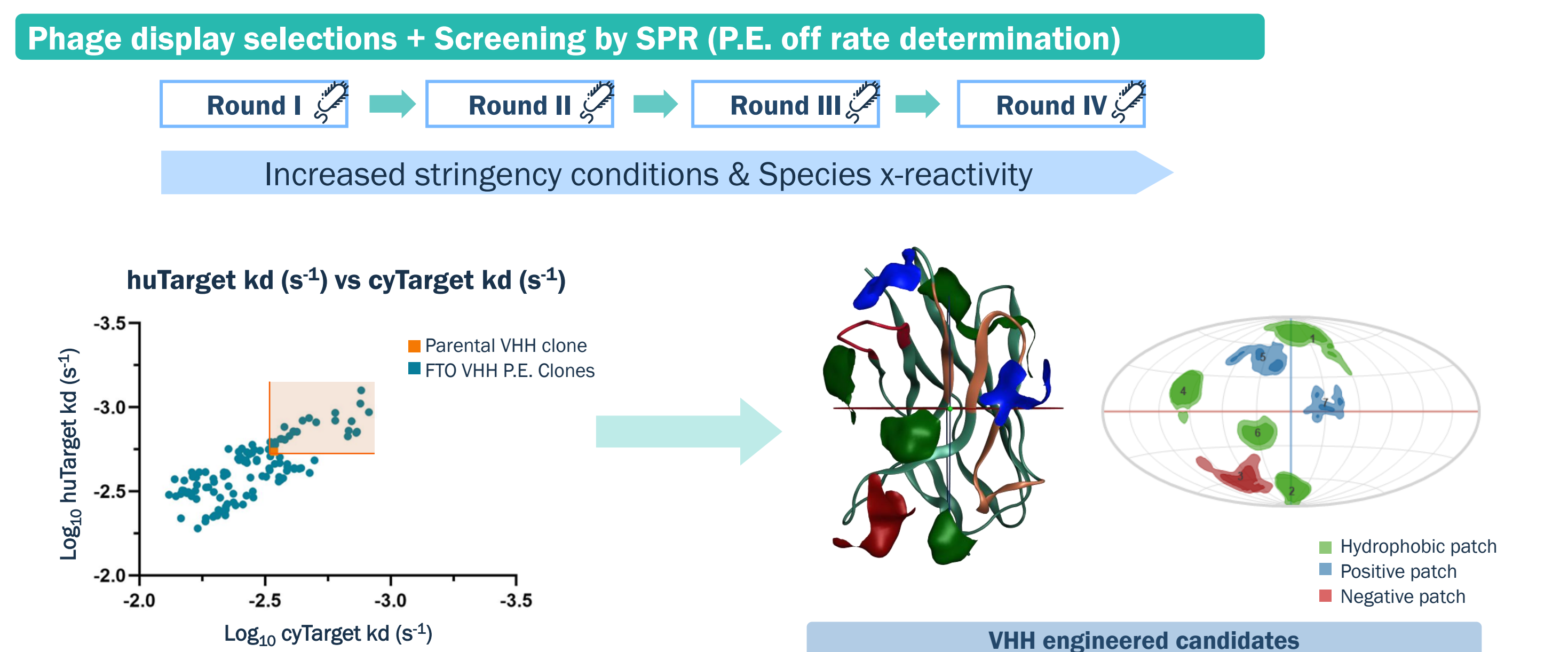
natural affinity maturation to generate high-quality binders with favorable biophysical properties. In this study, we implemented two complementary antibody discovery approaches targeting the same antigen: (i) an FTO-driven evolution of a parental VHH using targeted mutagenesis and display technologies, and (ii) a llama immunization campaign followed by library screening. By combining orthogonal strategies with early developability assessment, we aimed to maximize the likelihood of identifying robust, clinically relevant antibody candidates.



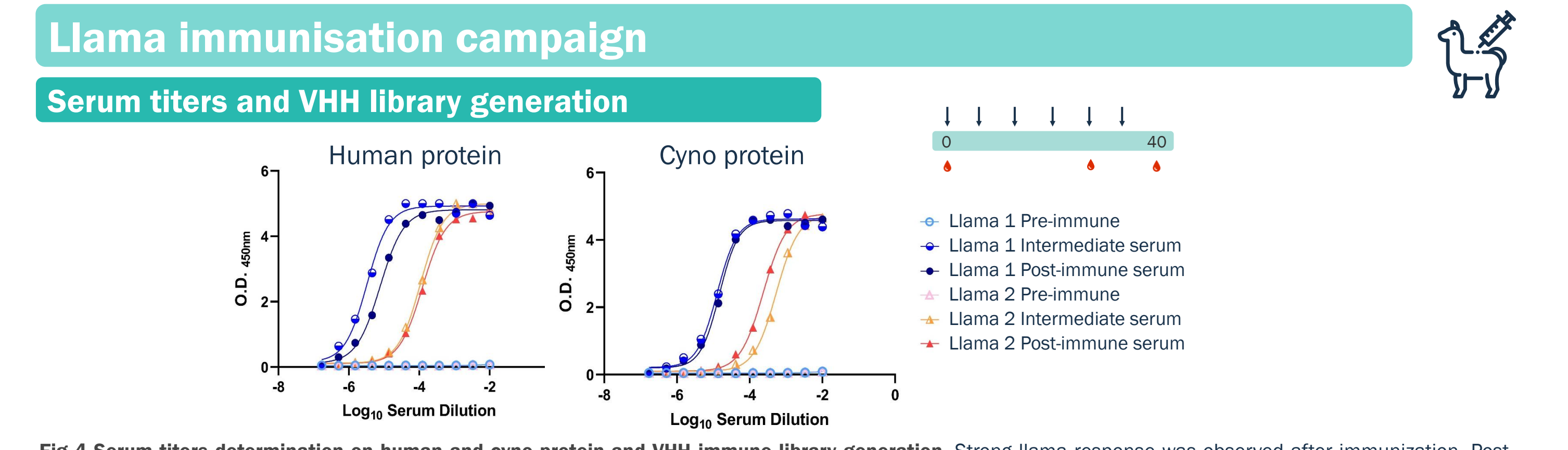
**Fig 1 Library design for VHH antibody** FTO evolution libraries were designed to mutagenize CDR residues as identified by Kabat and IMGT numbering scheme, and the predicted liabilities and developability issues identified by in silico risk assessment analysis (ISRA) of the parental antibody. One library per HCDR was generated.



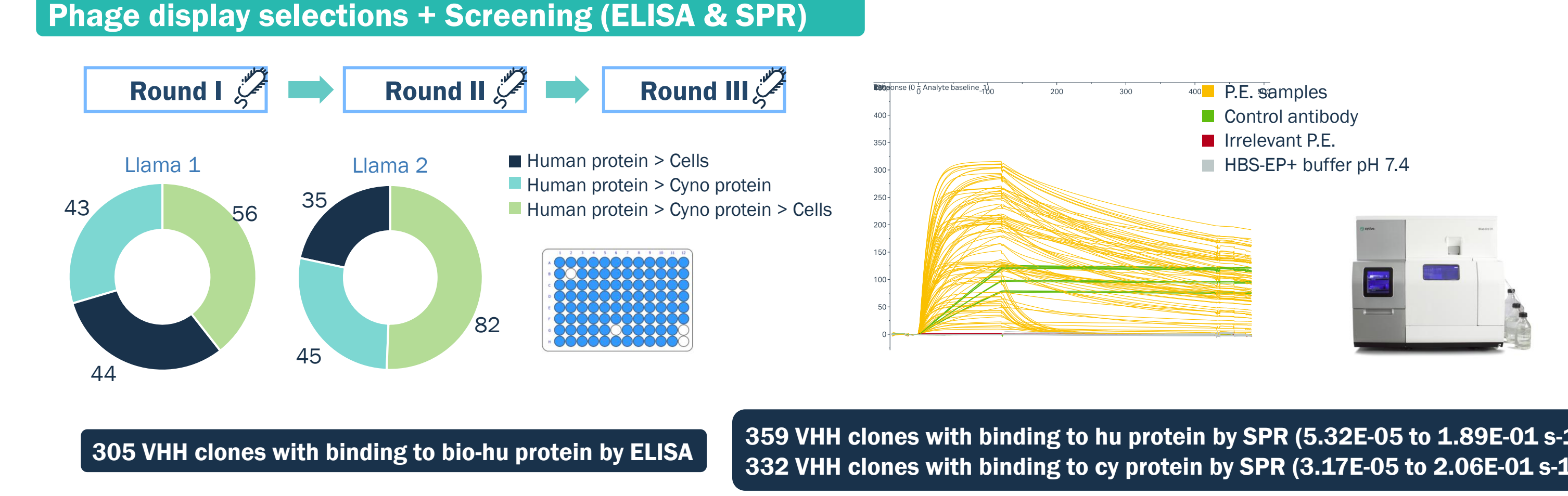
**Fig 2 NGS sequencing analysis from VHH antibody AFM libraries.** A. Average mutations per sequence: Normal distribution curve depicting the predicted and observed allowed mutations per sequence. B. Full sequence cluster diversity: Clusters were calculated at 100% identity. All sequences represented are high-quality and correct sequences. C. Amino acid distribution: Relative amino acid prevalence per Kabat position is shown, as counts. Designed and observed percentage WT residue are depicted in yellow and grey, respectively. D. Mutations per position: Percentage of sequences per designed mutate position (green) and not-designed region (red).



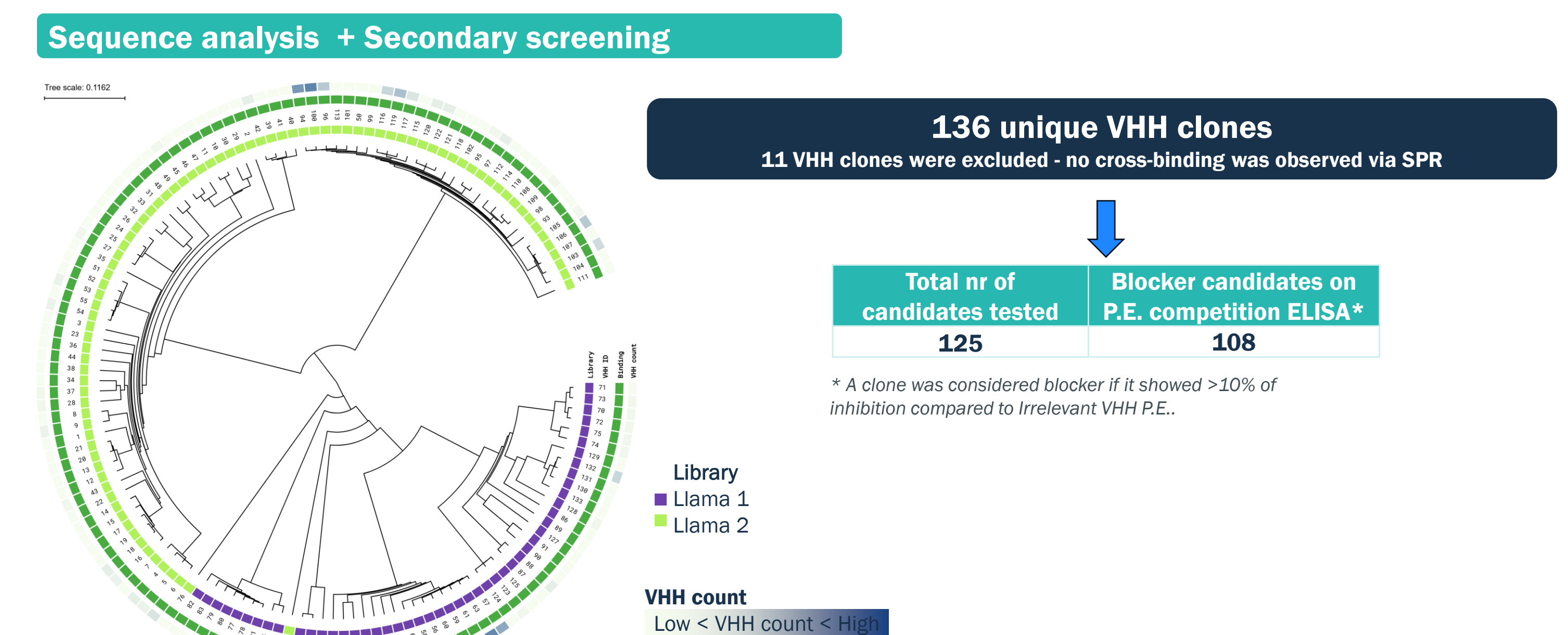
**Fig 3 Phage display selections, screening and selection of engineering lead panel.** After initial selection rounds on individual libraries, a combinatorial library was generated and further selected with increased stringent conditions. Selected clones were screened as Periplasmic extracts (P.E.) containing monoclonal VHH by SPR to determine the dissociation constant ( $k_d - s-1$ ). In the graph, it is possible to observe the correlation plot showing each individual P.E. sample with the calculated  $\log_{10} kd (1/s)$  for the human and cyno proteins reported in the y and x axis, respectively. Each VHH P.E. clone is represented as a blue dot and the parental VHH clone P.E. is represented as an orange dot. Clones falling inside of the upper-right orange box present a  $k_d (1/s)$  value lower than the VHH parental clone on both proteins. Clones with improved off-rate profile on both species were analysed by ISRA to de-select clones with potential liabilities/developability issues.



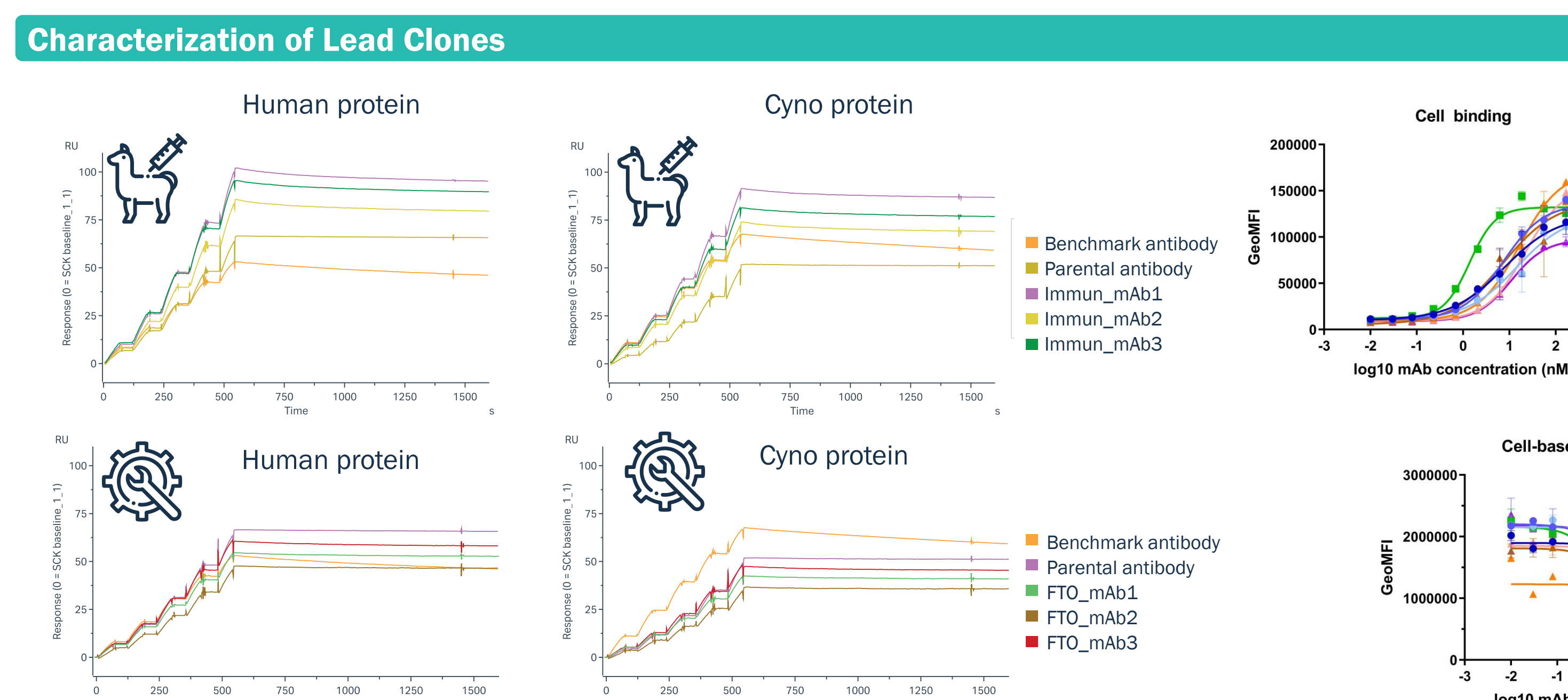
**Fig 4 Serum titers determination on human and cyno protein and VHH immune library generation.** Strong llama response was observed after immunization. Post-immune RNA samples from the immunized animals were used to generate phage display VHH immune libraries. These VHH immune libraries were used in phage display in-solution/cell selections.



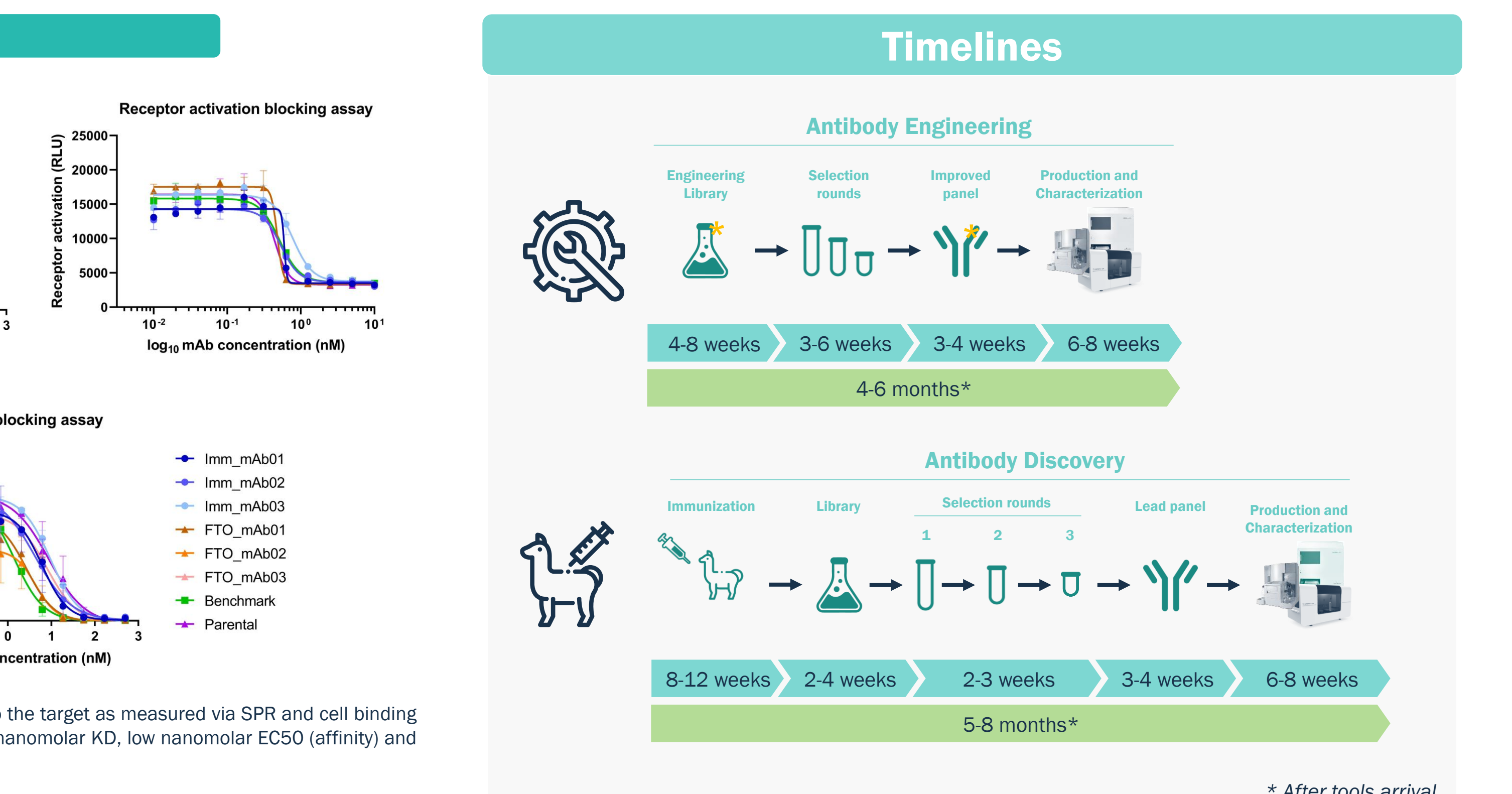
**Fig 5 Phage display selections and screening.** After three rounds of phage display in-solution and cell selections, selected clones were screened as Periplasmic extracts (P.E.) containing monoclonal VHH by ELISA and SPR to determine the dissociation constant ( $k_d - s-1$ ). 305 P.E. VHH clones showed binding to bio-human protein in ELISA while in SPR, 359 and 332 VHH clones showed binding to human and cyno proteins, respectively.



**Fig 6 Sequence analysis, secondary screening and selection of immune lead panel.** 136 VHH corresponding to unique sequences were identified, from which 125 showed cross binding between human and cyno proteins on SPR. 108 of these were shown to have blocking activity as P.E. samples via competition ELISA. ISRA analysis was also performed to assist in the selection of the lead panel for characterization.



**Fig 8. Characterization of small panel of purified lead antibodies.** The tested antibodies, from FTO evaluation and immune campaigns, show improved and/or similar affinity to the target as measured via SPR and cell binding FACS, and comparable potency as determined via a cell-based competition assay and via a receptor activation blocking assay using reporter cell lines. All clones showed sub-nanomolar KD, low nanomolar EC50 (affinity) and IC50 (inhibition potency) values.



### Conclusions:

Both FTO and llama immunization strategies successfully generated high-quality antibody candidates, with multiple hits matching or exceeding benchmark and parental antibody performance. Overall, no clear superiority was observed, highlighting the robustness of both approaches. FTO enables rapid, flexible in vitro discovery with precise control over antigen design and screening conditions, making it well-suited for challenging or time-sensitive targets. However, candidates may require additional optimization to achieve optimal developability. Llama immunization leverages in vivo affinity maturation, often yielding antibodies with strong stability and favorable biophysical properties. This comes at the cost of longer timelines, reduced experimental control, and reliance on animal immunization.

**Take-home message:** The two strategies are highly complementary—FTO offers speed and adaptability, while llama immunization provides naturally optimized binders. Combining both approaches can maximize diversity and increase the likelihood of identifying optimal lead candidates.