Accelerated evolution of nanobodies using DGRs

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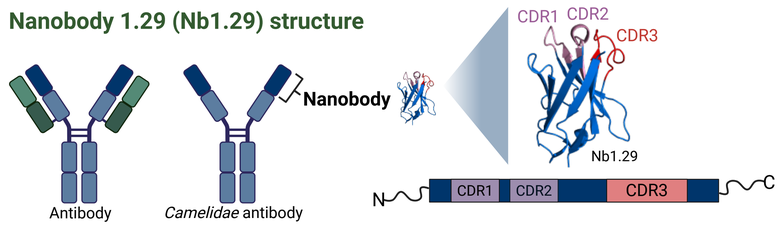
"We built a semi-continuous evolution platform to generate and select nanobody variants in E. coli "

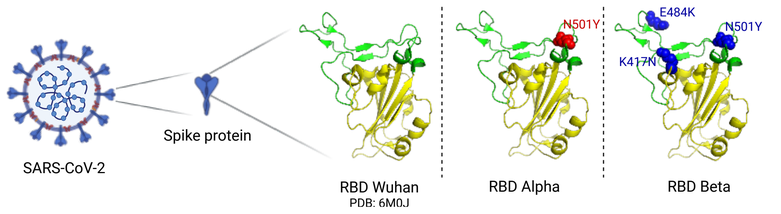
1. Background

Engineering proteins that bind specific targets is a major goal in bioengineering. **Nanobodies**—small antibody fragments derived from camelids—are especially attractive, but their development often relies on **animal immunization,** limiting scalability and speed. These challenges became particularly evident during the **COVID-19** pandemic, when the **rapidly evolving SARS-CoV-2** virus outpaced conventional antibody discovery pipelines. This highlighted the **need for faster, more adaptable platforms** to generate high-affinity binders in real time.

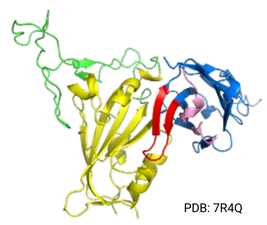
Nanobody 1.29 (Nb1.29) structure



SARS-CoV-2 and its variants

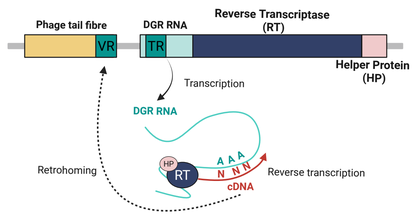


Nb1.29-Wuhan RBD complex

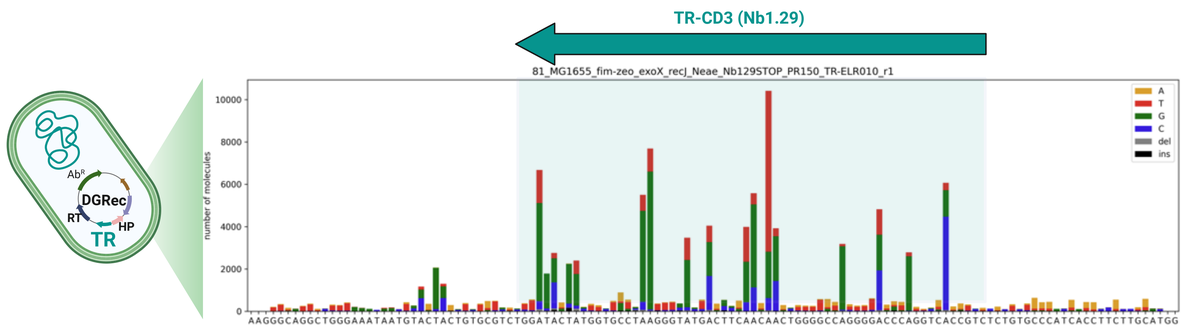


DGRec: teaching bacteria a phage trick

Diversity-Generating Retroelements (DGRs), first found in bacteriophages, diversify protein sequences *in-vivo*. DGRs **introduce mutations** during reverse transcription, particularly at **adenine** positions, enabling rapid exploration of the sequence space. **DGRec** is a synthetic system that **repurposes DGRs** to induce targeted and controlled hypermutation in *E. coli*.

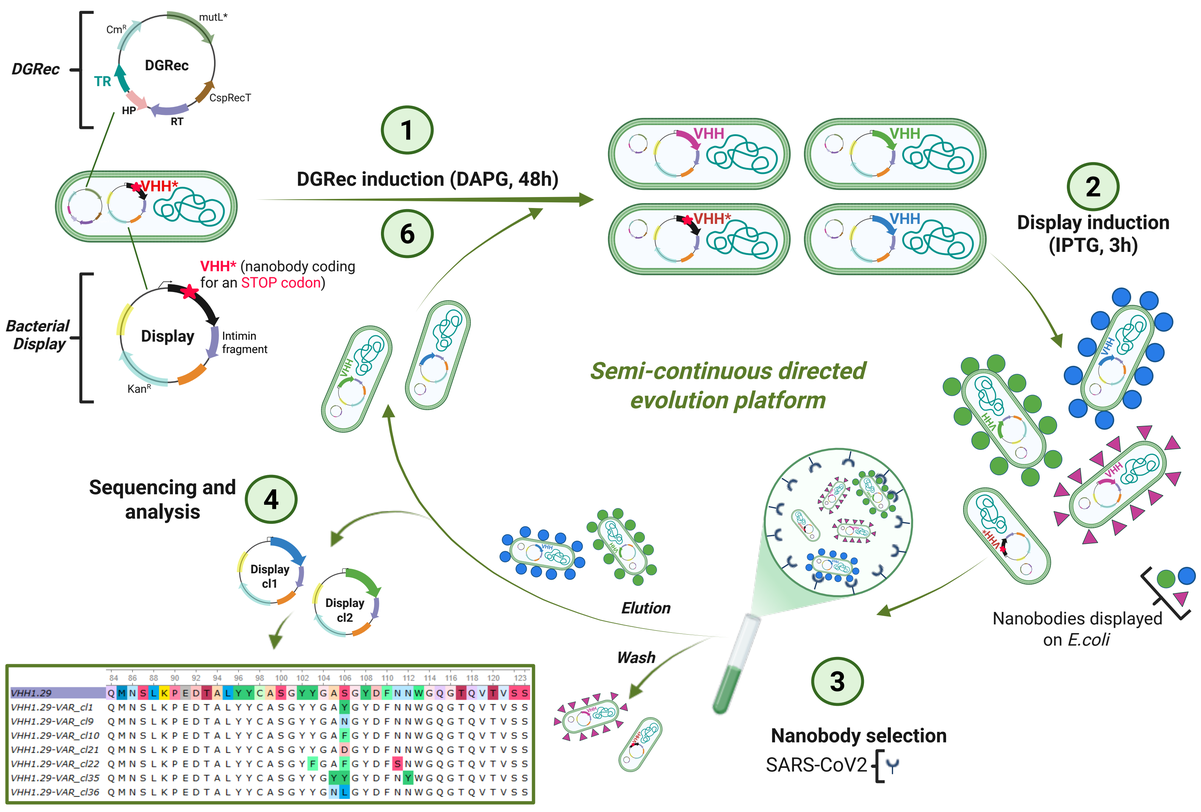


DGRec efficiently targets the CDR3 of Nb1.29 by specifying a template region (TR) to guide diversification.



2. Method development: Semi-continuous directed evolution platform

While DGRec provides a powerful system for generating nanobody diversity, the next challenge was selecting functional nanobody variants. To achieve this, we used **bacterial display**, which presents nanobodies on the surface of *E. coli*. This enabled **direct screening** of binding properties while retaining the underlying genetic information. Starting from an inactive form of Nb1.29 (S20*), we used the DGRec-bacterial display platform to try and isolate variants with **improved Beta binding**.

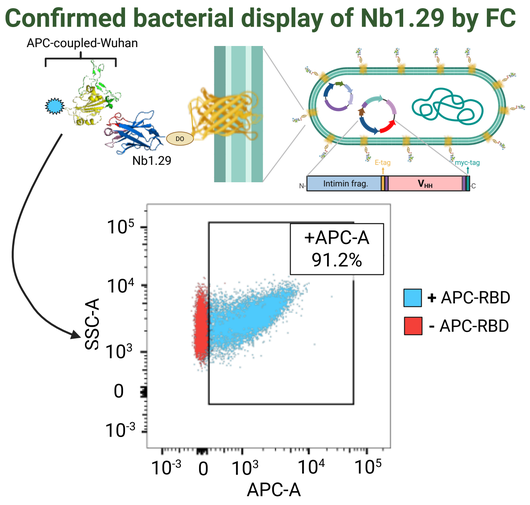


3. Proof of concept: Evolving Nb1.29* against SARS-CoV-2 variants

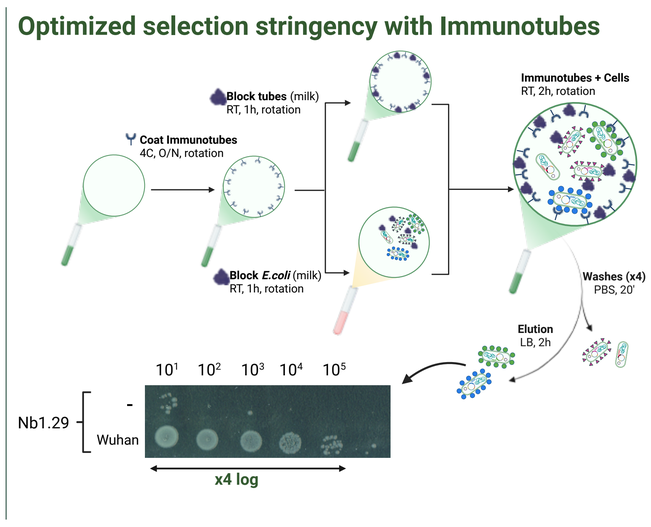
Assessing display and selection methods

We first needed to verify if the bacterial cells were **effectively displaying the nanobody**, and whether the **immunotube selection** was efficient.

Confirmed bacterial display of Nb1.29 by FC



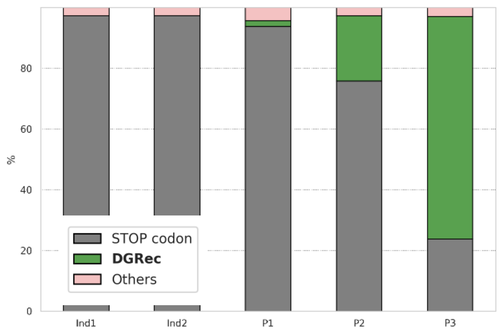
Optimized selection stringency with Immunotubes



DGRec profile evolution over time

Here we present the **nanobody-library genotypes** after Inductions 1 and 2, and Pannings 1 through 3. Following the inductions, the DGRec-derived population (green) comprised less than 1%. Selection against the Beta variant antigen led to **~80% of the population being DGRec-derived after P3**.

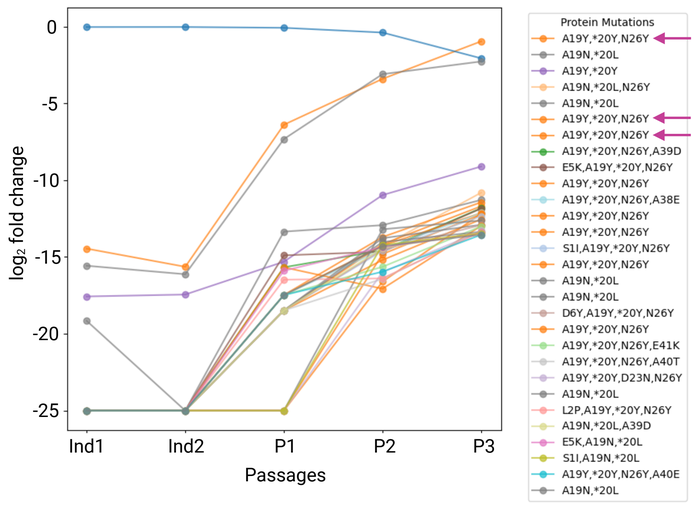
Nb1.29 CDR3 libraries panned against Beta



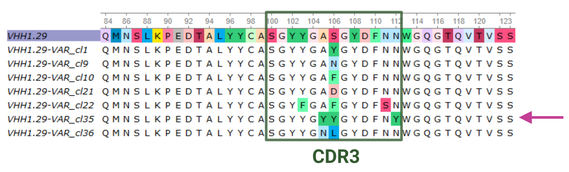
Identifying potential new binders at the protein level

We calculated the log2-fold change after each stage to identify protein **sequences** that were **enriched over time**. Proteotype (A19Y, *20Y, N26Y) **emerged independently** in multiple clones. To **understand the structural impact** of these mutations, we modeled the binding of this nanobody variant using AlphaFold Multimer. The mutations localized within the nanobody–RBD interaction interface, suggesting a **potential role in nanobody binding affinity.**

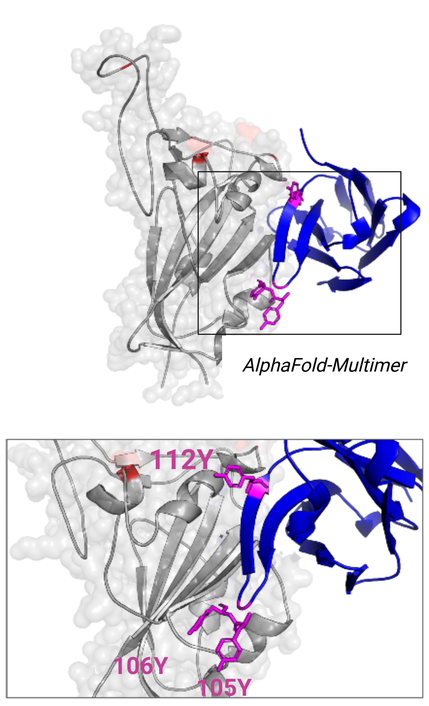
A) Detect "improved" Nb1.29 variants based on proteotype enrichment



B) Assess key residue positions across the nanobody sequence



C) Understand their potential effect at the structure level



4. Key takeaways

Our **semi-continuous platform** combining **DGRec and bacterial displa**y offers a powerful route to rapidly evolve **nanobody binders *in-vivo,*** bypassing the limitations of animal immunization.

This approach allowed us to:

- Successfully enrich SARS-CoV-2 binders from a non-functional starting nanobody
- Study genotype-phenotype linkage through nanobody evolution.
- Develop a preliminary framework for evolving other binding proteins beyond nanobodies

By integrating **robotic automation**, we will enable high-throughput protein evolution—a shift that could **accelerate protein discovery and optimization,** with far-reaching impact in synthetic biology, diagnostics, and medicine.

REFERENCES & ACKNOWLEDGMENTS

[1]Jovčevska, I., & Muyldermans, S. (2020). "The Therapeutic Potential of Nanobodies". BioDrugs, 34(1), [2]Casasnovas, J. M, et al., (2022). "Nanobodies Protecting From Lethal SARS-CoV-2 Infection Target Receptor Binding Epitopes Preserved in Virus Variants Other Than Omicron". Frontiers in Immunology, 13., [3] Molina, R. S et al., (2022). "In vivo hypermutation and continuous evolution". Nature Reviews Methods Primers, 2(1), 36., [4]S. Doulatov et al., (2004). "Tropism switching in Bordetella bacteriophage defines a family of diversity-generating retroelements," Nature, 431(7007).,

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