

# Engineering vaginal lactobacilli for continuous localized mucosal protection

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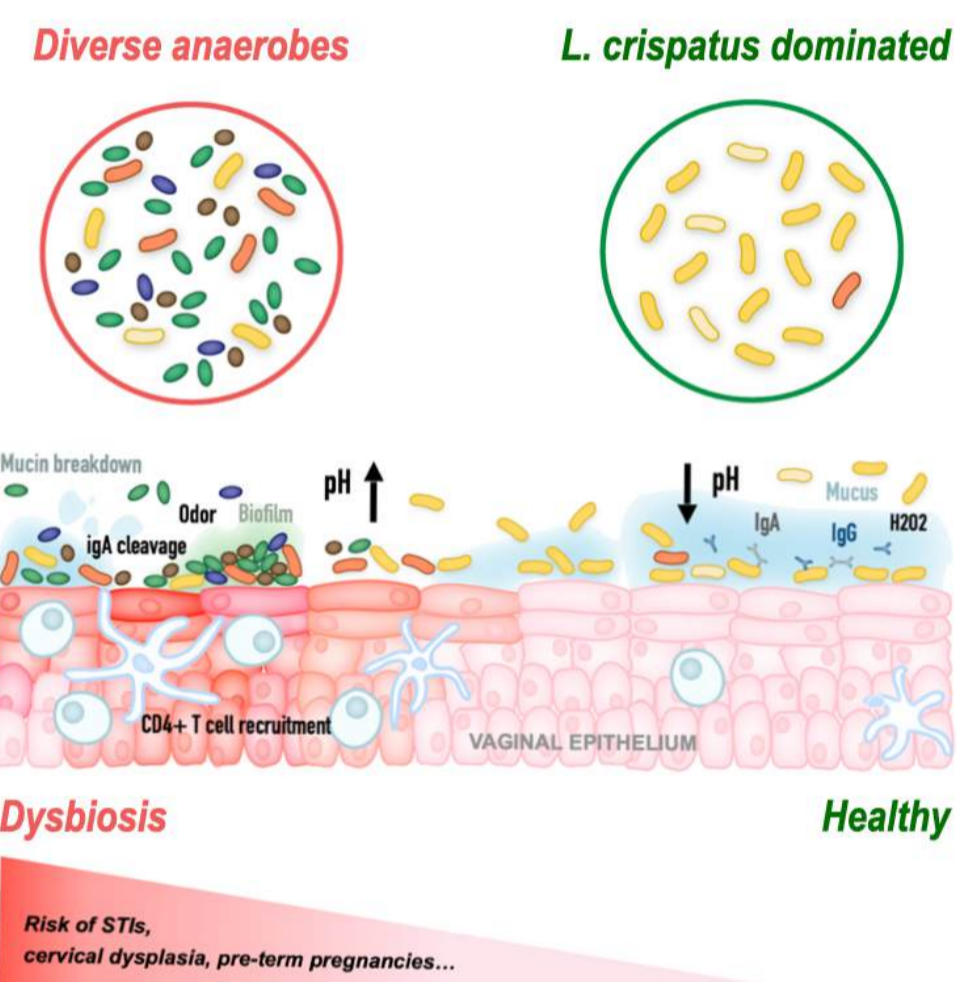
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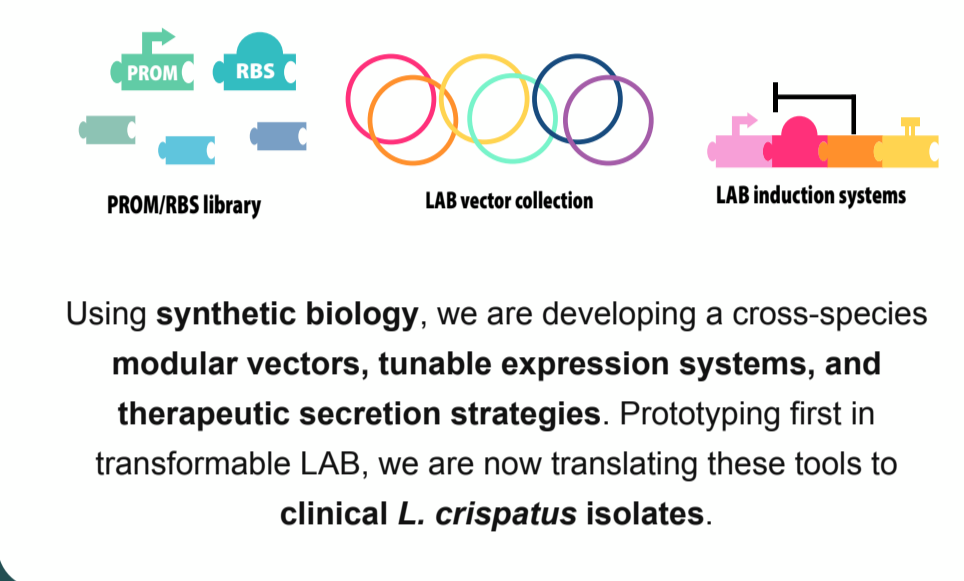
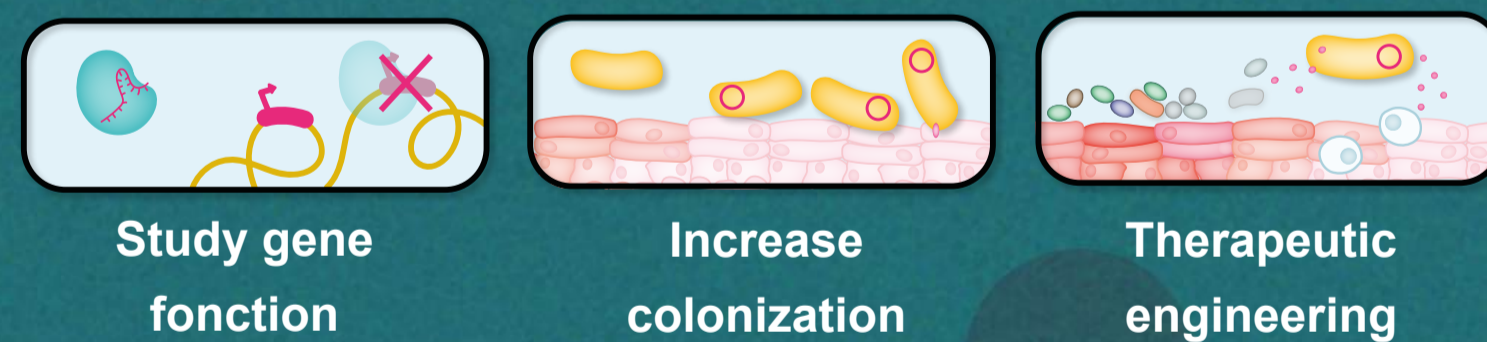
Lactic acid bacteria (LAB) are key chassis for **engineered biotherapies** due to their safety, host adaptation, and natural immunomodulatory potential. **Engineered LAB** are already explored mainly for cancer, gut and skin applications, **but progress is limited by the lack of standardized genetic tools, especially for non-model species.**



In the vaginal niche, *Lactobacillus crispatus* dominates **healthy microbiota** but remains **genetically intractable**, limiting its genetic study and vaginal therapeutic bacteria development...

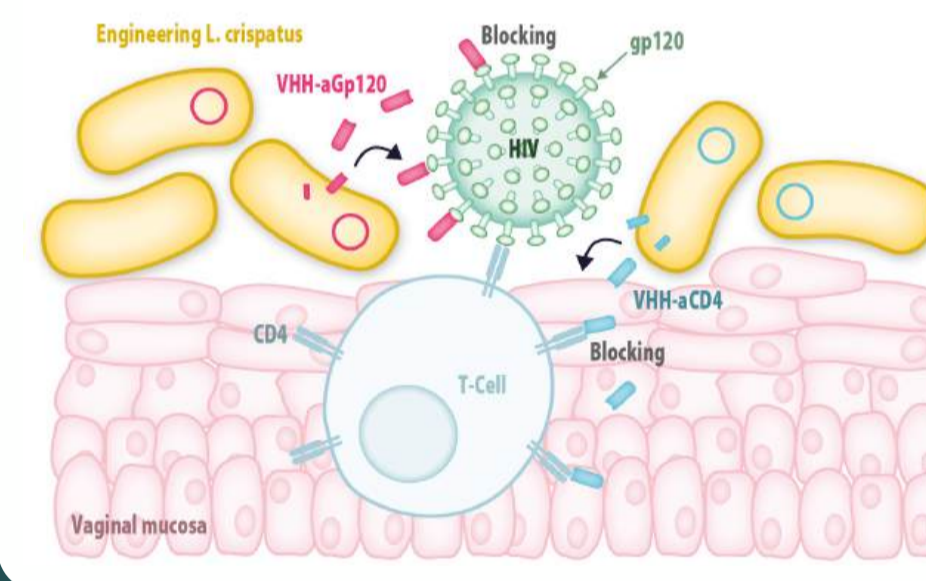


## POTENTIAL APPLICATIONS OF *L. CRISPATUS* ENGINEERING



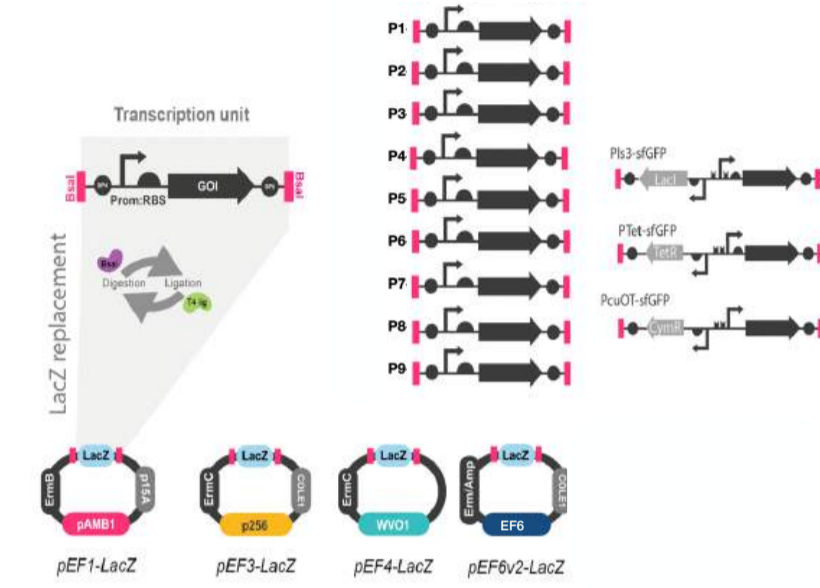
## Engineering an anti HIV probiotic ?

HIV remains a **major global health burden**, with young women in **Sub-Saharan Africa** disproportionately affected. Here, we propose using synthetic biology to **engineer next-generation vaginal probiotics** that **locally deliver anti-HIV nanobodies**, aiming to **reduce infection risk** in women at highest vulnerability.

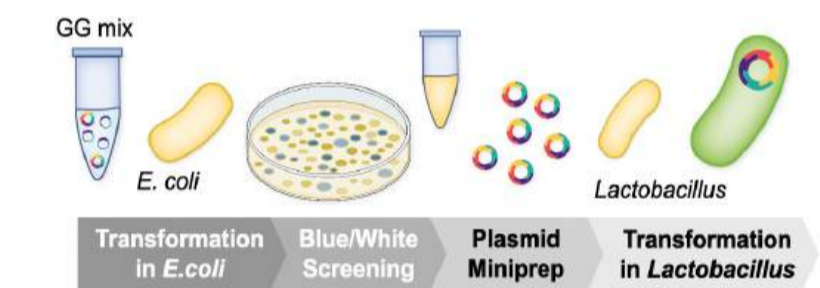


## A MODULAR PLASMID LIBRARY TO SCREEN PROMOTER:RBS FOR CONTROLLED GENE EXPRESSION

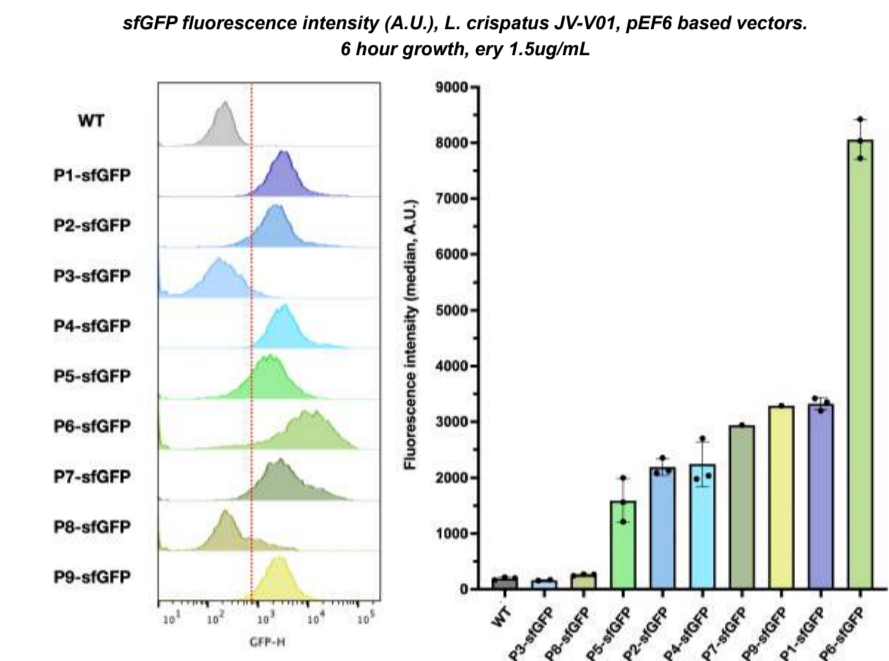
We designed a modular **Golden Gate** strategy enabling rapid assembly of promoter:RBS reporter plasmids across 4 compatible *E. coli*/*Lactobacillus* shuttle backbones. **9 constitutive expression cassettes** were designed (sGFP reporter) with promoter:rbs from both genome mining and already known sequences.



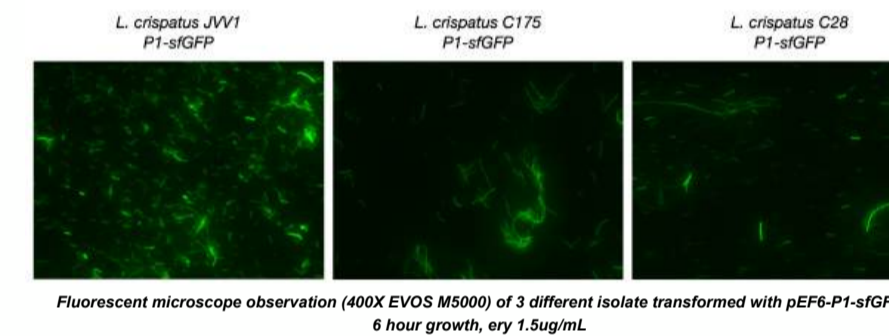
Reporter plasmids are first assembled in *E. coli*, then purified and transformed into *L. crispatus* clinical isolates for functional characterization.



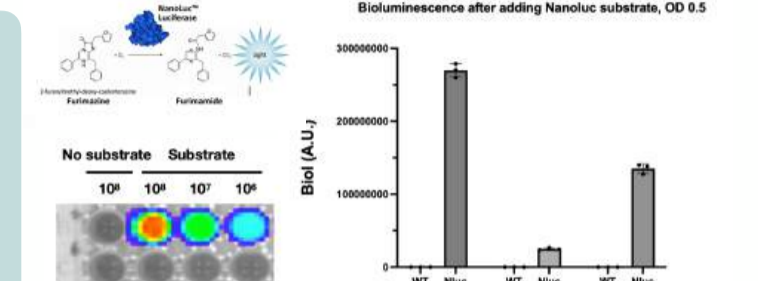
**Flow cytometry screening** reveals a **broad dynamic range** of sGFP expression driven by distinct promoter:RBS combinations in *L. crispatus*.



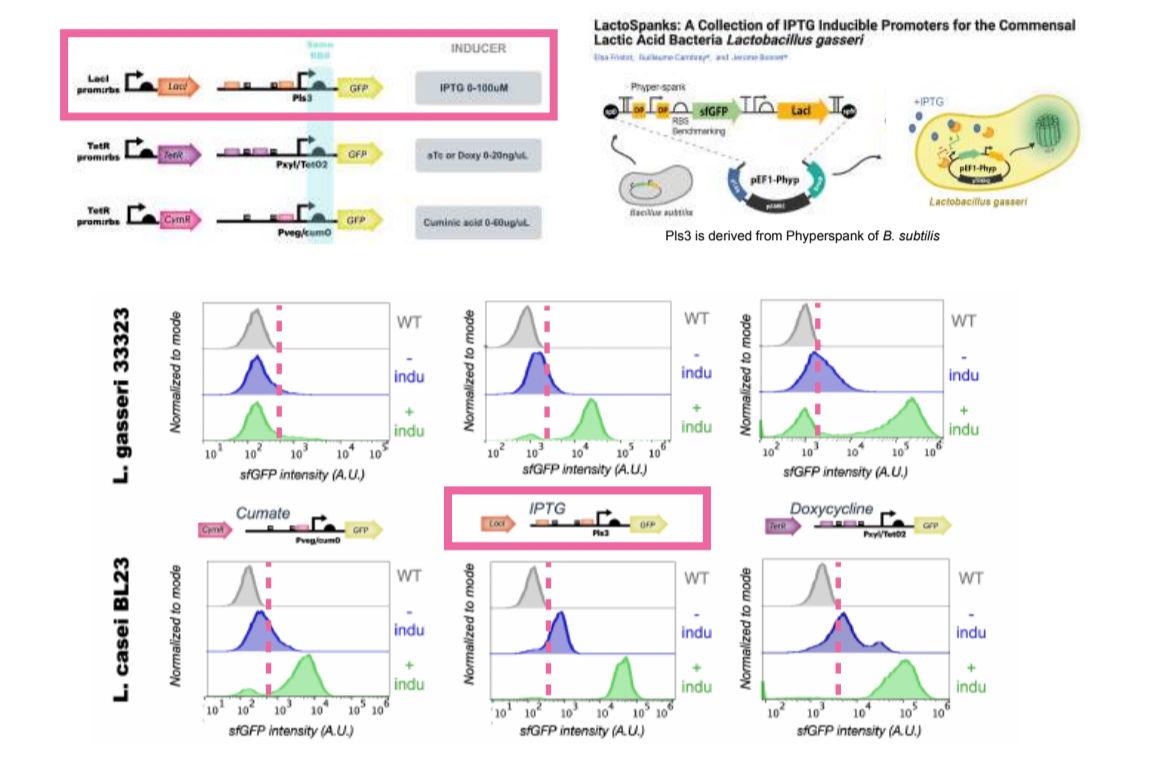
We generated a promoter:RBS library spanning **multiple expression levels** and validated reporter expression across genetically tractable *L. crispatus* clinical isolates.



In preparation for future in vivo studies, we engineered **bioluminescent *L. crispatus*** strains, expressing **Nanoluciferase**, to enable longitudinal monitoring of vaginal colonization.

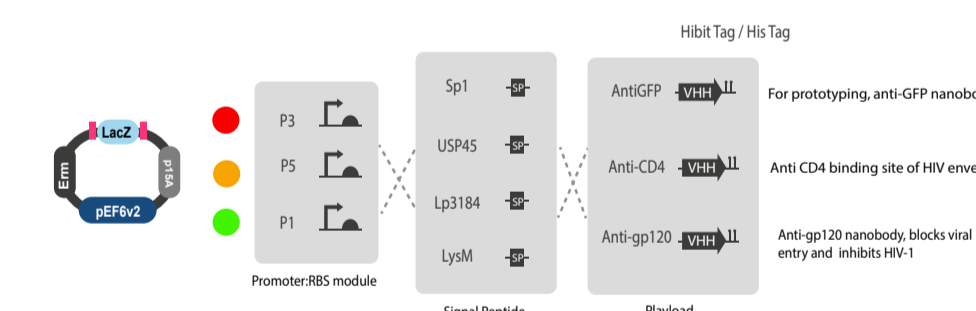


To achieve precise control of gene expression in lactobacilli, we developed standardized, in vivo-compatible **inducible expression cassettes** and evaluated their functionality in *L. gasseri* and *L. casei*. In *L. casei*, all three systems enabled strong sGFP expression upon induction. In *L. gasseri*, the cumate system showed low expression and requires further optimization.

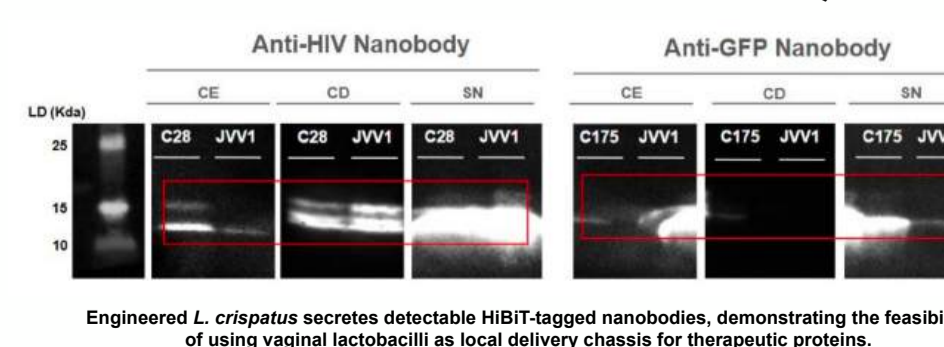
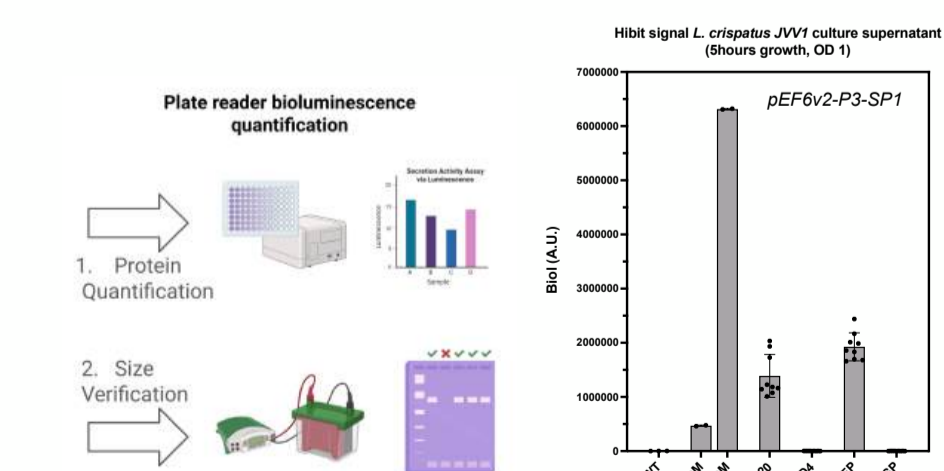


## SECRETION OF ANTI HIV NANOBODIES

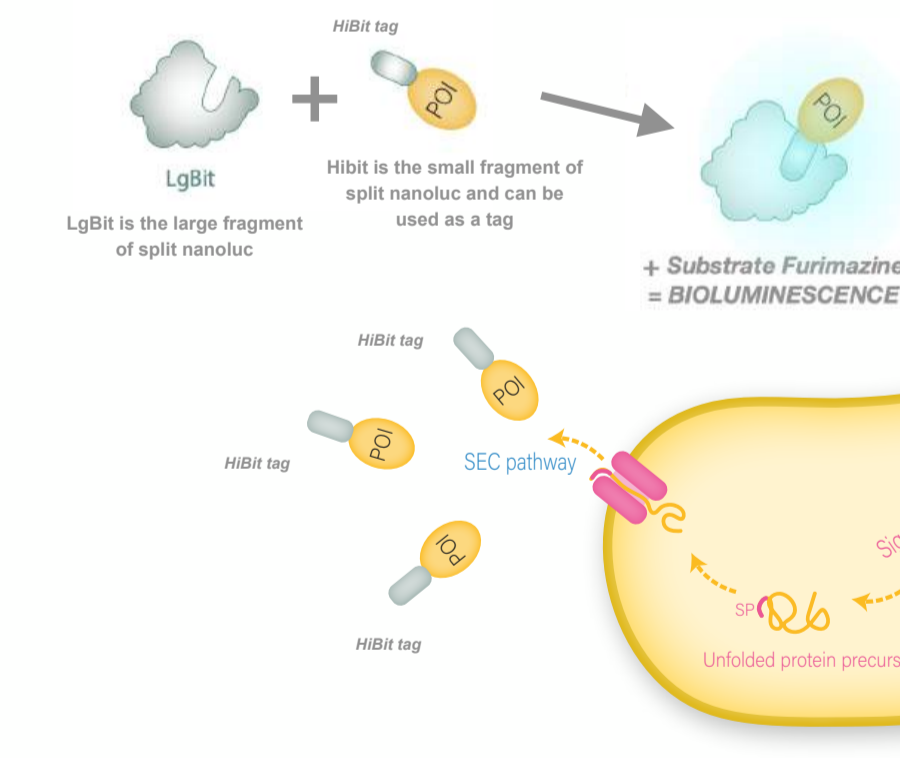
To enable rapid prototyping of therapeutic nanobody secretion, we tested different combination of Promoter:RBS modules, signal peptides and HIBIT/His-tagged nanobody payloads, including anti-GFP for secretion benchmarking and anti-HIV candidates targeting CD4-binding or gp120-mediated viral entry.



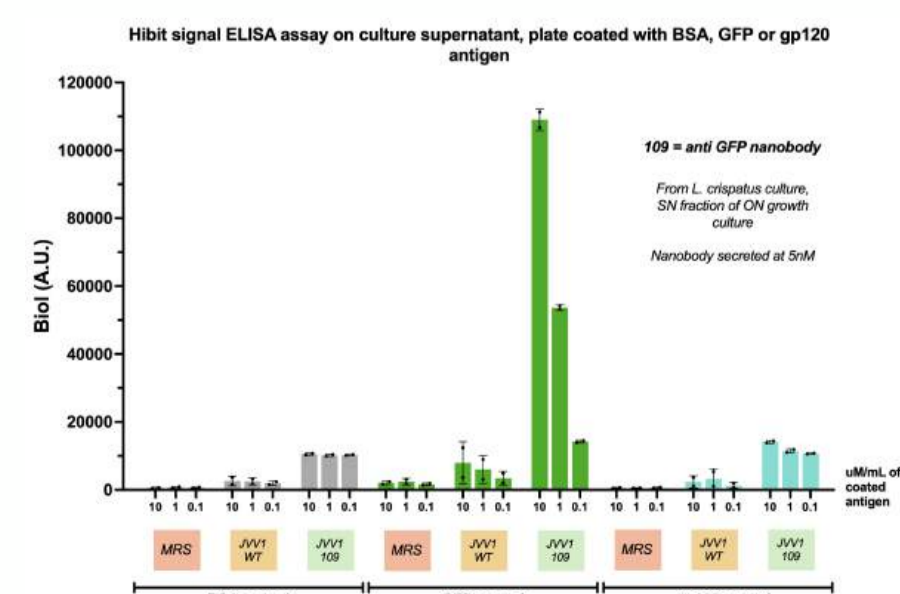
Among all combinations tested, **pEF6v2-P3-SP1** constructs carrying the different nanobody payloads were the **only ones that passed both sequence verification and transformation into *L. crispatus***. Secreted proteins were then quantified using a plate-reader bioluminescence assay based on HIBIT/LgBIT complementation and validated by gel-based size verification. Anti-HIV gp120 and anti-GFP nanobody constructs produced detectable extracellular signals in culture supernatants, confirming that **engineered *L. crispatus* can secrete therapeutic protein candidates**.



We adapted the **split NanoLuc HIBIT system** to monitor secretion of recombinant proteins: therapeutic payloads are fused to a signal peptide and HIBIT tag, exported through the Sec pathway, and detected by complementation with LgBIT and furimazine.

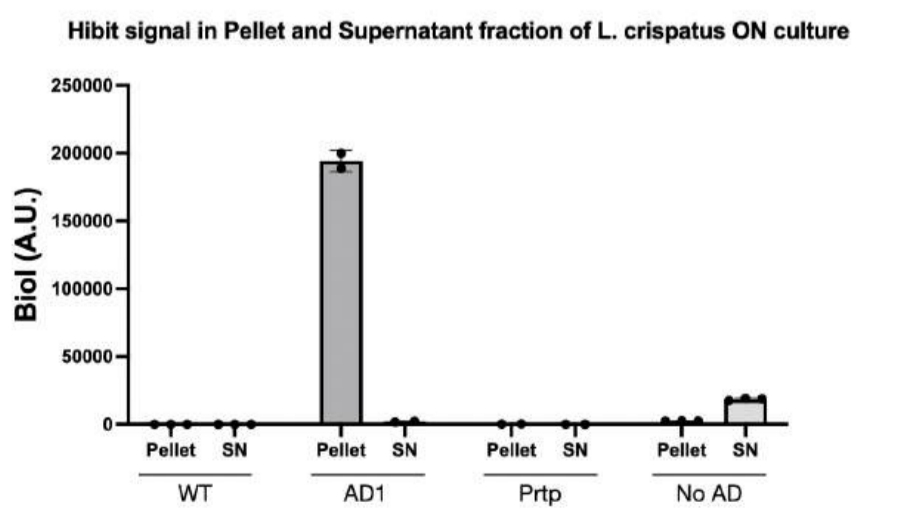
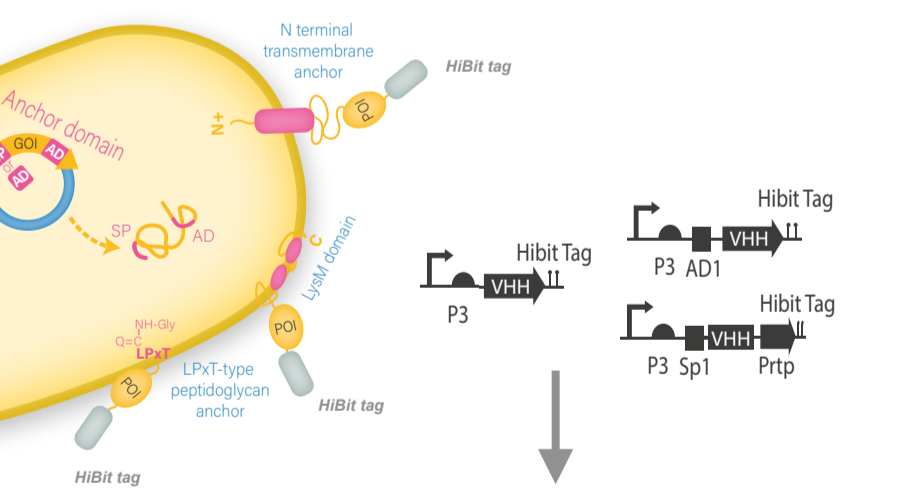


Culture supernatants from *L. crispatus* JV-V01 expressing the HIBIT-tagged anti-GFP nanobody showed strong, antigen-specific signal on GFP-coated plates, with minimal signal on BSA or gp120 controls. This confirms that the **secreted nanobody is not only detectable in the supernatant, but remains functionally folded and capable of target binding after secretion**. Our next step is to test the binding of the secreted anti-gp120 nanobodies.



## MEMBRANE DISPLAY

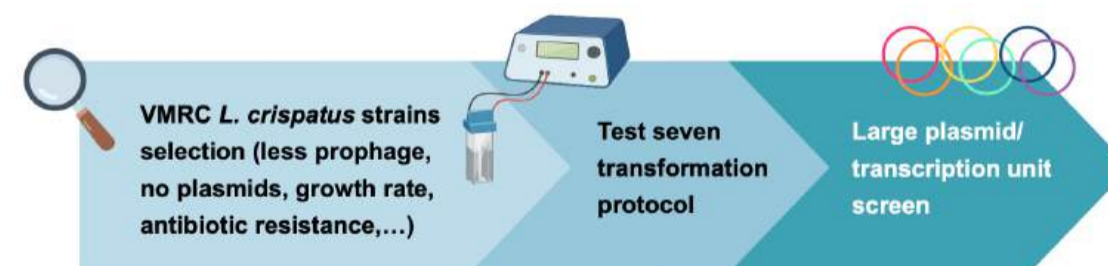
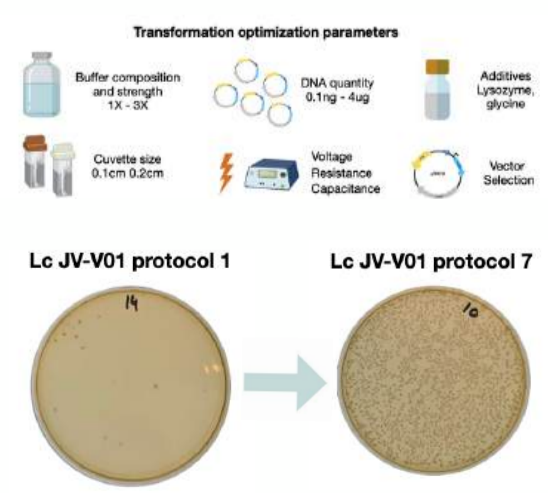
While secretion of therapeutic is primordial in our project, we also wanted to develop the **membrane display** of those same proteins. With a similar approach, we designed membrane display module in which proteins of interest (**VHH anti GFP, for prototyping**) are fused to a signal peptide, a HIBIT detection tag, and candidate anchoring domains, enabling comparison of surface-retention strategies in engineered *L. crispatus*.



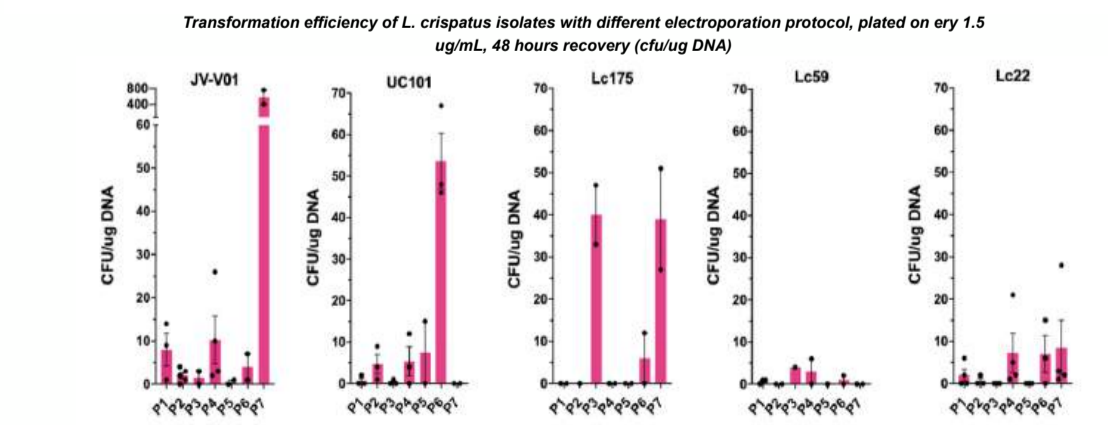
HIBIT signal detected in pellet and supernatant fractions confirms **anchor-dependent localization** of displayed anti GFP nanobody. The AD1 construct shows strong cell pellet-associated signal, consistent with efficient membrane or cell-surface retention. **We will soon test the binding efficiency of the displayed protein using purified GFP.**

## OPTIMIZING ELECTROPORATION FOR *L. CRISPATUS* CLINICAL ISOLATES

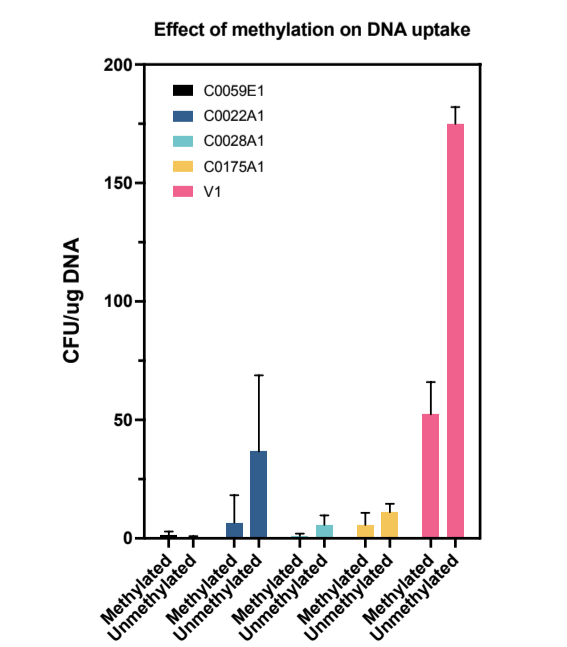
Despite being a key member of the vaginal microbiota, *L. crispatus* remains **largely unengineered**, mainly due to its **low transformability**. This likely results from restriction-modification systems, phages, its cell envelope or plasmid incompatibility. **Optimizing electroporation conditions** is therefore essential to identify compatible isolates and improve transformation efficiency.



**Workflow to assess *L. crispatus* transformation potential**



Plasmid methylation strongly impact DNA uptake in selected *L. crispatus* isolates, as shown by comparing plasmid DNA extracted from NEB 10-beta (methylated) versus dam/dcm (unmethylated) *E. coli*, highlighting restriction-modification barriers as a major bottleneck for transformation.



We tested 7 modified electroporation protocols across multiple clinical *L. crispatus* isolates using pMG36e or pEF6-mRFP as screening vectors. The optimized pEF6-based protocol yielded the highest transformation efficiency in strain JV-V01, reaching **7 x 10<sup>8</sup> CFU/ug DNA**.