# Engineering phages for eukaryotic payload expression in cancer associated *Fusobacterium Nucleatum*

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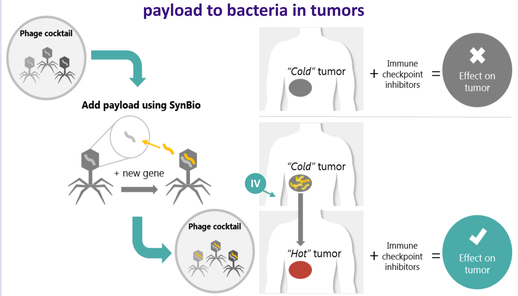
## Background

*Fusobacterium nucleatum* (*FN*) is enriched in human colorectal tumors [1] and its presence is correlated with poor prognosis [2]. Bacteriophage ("phage") have high intrinsic safety and specificity and offer a promising treatment strategy to target *FN* associated with colorectal cancer (CRC). Phage engineering allows specific delivery of anti-tumor immune stimulating payloads. Phage targeting FN have been isolated. A major challenge in engineering these phage is to express eukaryotic payloads in non-model clinical prokaryotic hosts, such as *FN*.

**Engineered phage designed to bring immune-stimulating payload to bacteria in tumors**



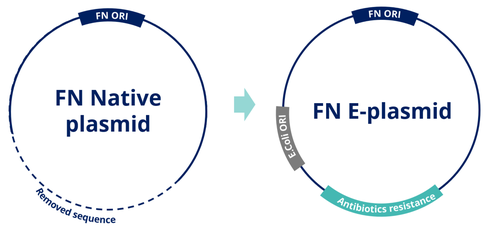

## Highlights

- We demonstrated the ability to express a eukaryotic protein in an unconventional bacterial host using a sequence designed by computational tools
- Our engineered phage can deliver a eukaryotic payload to its host with a similar pattern as an expression vector
- This methodology suggests that expression systems can be introduced by phage engineering into specific clinically relevant bacteria

## 1 Generating a molecular system *de-novo*

### Designing a shuttle vector from a native plasmid

A shuttle vector was generated using a native *FN* plasmid, by adding an *E.coli* ORI and an antibiotic resistance gene for selection. Following this, the plasmid was shortened by knocking out unnecessary sequences.




### Finding a strong promoter for expression

Mass-spec analysis of *FN* bacteria lysates

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Selection of highly expressed proteins

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Cloning the selected genes with their promoters and a luminescent-tag (HiBiT) into the *FN* E-plasmid

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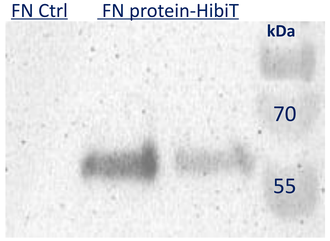
Expression in *FN* and validation by WB

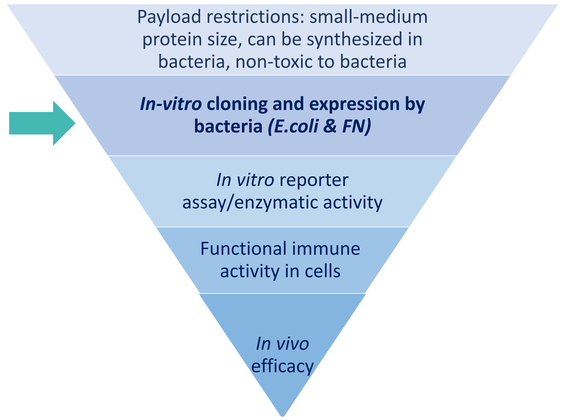
Expression cassette in *FN* E-plasmid


Western blotting (WB) of *FN* lysates expressing an endogenous protein conjugated to a luminescent-tag (HiBiT, Promega) cloned in *FN* E-plasmid. *FN* Ctrl - control samples without a plasmid.

**Following validation of expression, the endogenous protein's gene can be replaced with a payload of interest**

## 2 Selection process for potential payloads



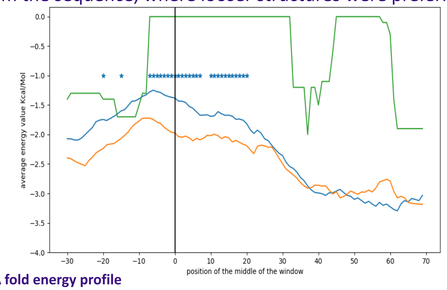

One of the selected payloads was **Interleukin 15 (IL15)** – a cytokine that stimulates the proliferation and cytotoxic function of CD8+ T cells and NK cells.

## 3 Optimizing expression with computational tools

Working with non-model clinical bacteria is a challenge due to the lack of data on codon usage and non-coding genetic information. To address these challenges, two main computational approaches were utilized:

**1. Mimicking host transcriptome:** The final construct is built out of a string of large stretches of adjacent codons which can also be found in the host transcriptome.

**2. mRNA fold energy at translation initiation vicinity:** Codon selection in the area of translation initiation was guided by the local mRNA secondary structure resulting from the sequence, where looser structures were preferred.




**The average RNA fold energy profile**

X axis is the position on the coding sequence of genes and the Y axis is the RNA folding energy. **Blue** and orange lines show the energy profile for the 100 highest and least expressed genes in *FN* (respectively). The Green line is the average RNA fold energy profile of the optimized IL15 gene. Blue stars mark significant difference between the energy of the highest and least expressed genes.

## 4 Validation of eukaryotic protein expression in *FN*

To test expression of the optimized IL15 gene sequence in *FN*, the IL15 gene was introduced on our E-plasmid and validated by WB and luminescence assay.

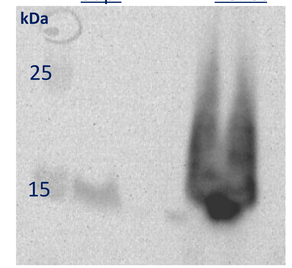
The luminescent signal was obtained from the HibiT-tag conjugated to the IL15 protein using a commercial kit (Promega).

The payload was observed in both fractions of the FN lysate. Following this, the payload was ready for engineering into an FN phage.

A

| Sample | Lumi. signal (RLU) |
|---|---|
| FN+IL15 | 112,117 |
| FN Ctrl | 37 |

B




**(A)** Luminescence assay and **(B)** WB of *FN* expressing IL15 conjugated to a luminescent-tag (HiBiT) cloned in *FN* E-plasmid.

## 5 Payload expression following engineered phage infection

Generating engineered phage via a synthetic biology approach

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Isolating the engineered phage

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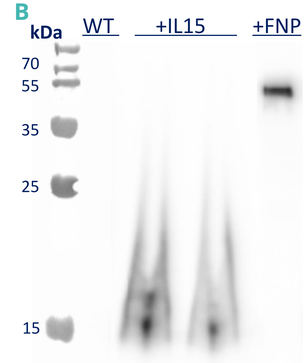
Propagating the engineered phage on *FN* host

A

| Sample | Lumi. signal (RLU) |
|---|---|
| Engineered phage lysate | 3,140,905 |

B




**(A)** Luminescence assay and **(B)** WB of *FN* lysate after engineered phage infection. **WT** – wild type phage, **IL15** – phage genome contains IL15, **FNP** – phage genome contains same endogenous *FN* protein as previously shown.

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## References

[1] Ito M, et al. Association of Fusobacterium nucleatum with clinical and molecular features in colorectal serrated pathway. Int J Cancer. 2015; 137:1258–1268.
[2] Yu T, et al. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. Cell. 2017; 170:548-563.