SYN SENSO

Cell-free synthetic biology for combinatorial biosensor design

Project no. 101072980

Deliverable D1.3
Novel RBPs for translational control

Version 1.1

WP 1 – Building cell-free computing circuits

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Revision history

Date	Authors	Revision
18/02/2024	Dudu Boyvat	Draft version
20/02/2024	Velia Siciliano	First version
28/02/2024	Heinz Koeppl, Christiane Hübner	Internal review
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Contents

Abbreviations

Abbreviations	Details
EGFP	Green fluorescent protein
HIV	Human Immunodeficiency Virus
NC	Negative control
RBP	RNA binding protein
RRL	Rabbir reticulocyte lysate
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
TCS	TEV cleavage sequence
TEV	Tobacco Etch Virus
TEVp	Tobacco Etch Virus protease
TVMV	Tobacco Vein Mottling Virus
TVMV-CS	Tobacco Vein Mottling Virus protease
WT	Wild type

Partner Short Names

Abbreviations	Details
TUDa	Technische Universität Darmstadt
TUE	Eindhoven University of Technology
IIT	Istituto Italiano di Technologia
TVU	Tor Vergata Universita degli Studi di Roma
DBS	Dynamic Biosensors GmbH
UBI	Ulisse Biomed S.p.A.
ICL	Imperial College London
ABV	Abvance Biotech S.L.
ACC	accelopment Schweiz AG
LEB	LenioBio GmbH
BRA	BRAIN Biotech AG
NUC	Nuclera Nucleics Ltd.
BME	Biomerieux SA
UNA	University of Naples Federico II

Executive Summary

This Deliverable is a plan for selecting RNA-binding proteins (RBP) and re-engineering for post-transcriptional control and identifying the viral-specific proteases and their cleavage sites (i.e., HBV, HCV, HIV, SARS- CoV-2).
MS2-CNOT7 functions as an RNA-binding protein and can undergo engineering by the insertion of different sequences. To initiate our investigations, MS2-CNOT7 was selected for assessing its interactions with human-infective viral proteases. Initially, plasmid constructs were created to evaluate the MS2-CNOT7 with human-infective viral proteases within cellular environments, given that its activity has been previously verified in vivo. While repression of MS2-CNOT7 was observed with the newly designed constructs, de-repression via human-infective proteases could not be achieved. Subsequent in vivo experiments will involve testing within cells infected by a collaborator's viral strain. Subsequently, the MS2-CNOT7 system underwent in vitro evaluation. The primary objective was to observe MS2-CNOT7 repression within a cell-free system followed by testing de-repression. To ensure optimal control, a mutated MS2-CNOT7 construct was cloned. However, analysis of the data and previous studies on our cell-free lysate in the literature revealed that the presence of a polyA tail, which is crucial in cellular systems, does not exert a significant influence within our cell-free environment. Furthermore, L7Ae, which is another RNA-binding protein, will be tested in a cell-free system.

Need for the Deliverable

Re-engineering an RNA-binding protein MS2-CNOT7 for post-translational control and testing in cell-free.

Objectives of the Deliverable

- Testing MS2-CNOT7 system on cell-based system for human infective viral proteases.
- Testing MS2-CNOT7 system on cell-free system to prove post-translational control ability.

Outcomes

- MS2-CNOT7 RNA binding protein shows promise as a suitable candidate as a biosensor component in cellular systems.
- MS2-CNOT7 system has been tested in a cell-free system but does not show any repression on translation.
- Poly-A tail is not important for the reporter in the cell-free lysate
- The RNA de-adenylation process is not an ideal candidate for the design of biosensors in cell-free systems

Next steps

- L7Ae is another RNA-binding protein. It has the ability to bind kink-turn on RNA.
- Reporter luciferase will be cloned with a 2 kink-turn structure.
- L7Ae and reporter luciferase will be cloned under the T7 promoter
- The system will be tested in rabbit reticulocyte lysate.
- After showing the repression, the L7Ae protein will be re-engineered to be tested with human-infective viral proteases.

1 Introduction

RNA-encoded regulatory circuits are crucial elements for reprogramming cellular function and, with some limitations, are promising as they eliminate the risk of the DNA-based system. Here, the RNA-binding protein MS2-CNOT7 is tested to recognize protease activity. MS2 is a bacteriophage coat protein that binds small RNA loops in viral RNA while CNOT7 is a de-adenylase protein. MS2-CNOT7 can effectively block the translation of an RNA carrying MS2 binding loops. Briefly, the MS2 protein binds to the loops and brings the CNOT7 in close proximity to the poly-A tail of an mRNA. CNOT7 deadenylates and un-stabilizes the reporter mRNA causing degradation.

This principle can be applied to viral infection detection. Inserting a specific cleavage site targetable by a viral protease between these two proteins can make the system recognize viral protease availability in the environment. Here, we would like to develop a cell-free platform based on an RNA-binding system for the detection of viruses within humans.

MS2-CNOT7 chimeric protein is tested in the cell-based system in literature with different, non-human infective viruses. Since cell-based assays are optimized systems to confirm the MS2-CNOT7 activity, here, MS2-CNOT7 is tested in a cell-based assay for TEV (Tobacco Etch Virus) and TVMV (Tobacco Vein Mottling Virus) viral proteases. Briefly, specific cleavage sequences for TEV and TVMV proteases are inserted between MS2 and cNOT7 proteins. 8 MS2 loops were cloned to the 3'UTR of a reporter green fluorescent protein (EGFPx8MS2). When MS2-CNOT7 chimeric protein is transfected to cells together with EGFPx8MS2, the system represses the EGFP translation. If viral protease is available, it cleaves the specific site and releases the system from the RNA, and the translation of EGFP is rescued.

Further, MS2-CNOT7 chimeric protein is engineered to detect human-infective viruses. Three different human-infective viruses (SARS-CoV-2, HIV-1, and Dengue) have been chosen and from the literature specific cleavage sequences are obtained. MS2-CNOT7 protein is engineered to have a specific cleavage site between two proteins as a linker. For proteases, encoding plasmids are ordered on Addgene for the following viruses, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), dengue, and human immunodeficiency viruses (HIV).

The main objective here in this project is to implement an RNA-binding protein biosensor system to the cell-free. Cell-free systems have fast development times compared to cell-based assays. Also, resistant to the study of toxic input material which here is an important advantage to test protease activity.

RNA-binding protein MS2-CNOT7 tested in a linked cell-free system. Initially, requisite sequences were cloned downstream of the T7 promoter. With PCR, coding sequences are amplified with a poly-A tail. Subsequently, RNA synthesis ensued utilizing the T7 MEGAscript kit from PCR templates, while rabbit reticulocyte lysate (RRL) facilitated protein generation. RRL is a cell-free system that uses RNA as a starting template. The selection of RRL, a cell-free system reliant on RNA, stemmed from the centrality of RNA-binding proteins in the project, particularly emphasizing the essential requirement for precise control over the intricate interplay between reporter RNA and RNA-binding protein.

One drawback of this system relates to the color of the lysate, derived from immature red blood cells of rabbit reticulocytes, necessitating the substitution of our reporter EGFP with luciferase due to potential interference between green and red colors.

2 Cell-Based Experiments

2.1 Testing TEV and TVMV protease activity with MS2-CNOT7 system

To assess the functionality of the MS2-cNOT7 system, a series of plasmids including MS2-TCS-cNOT (where TCS represents the TEV cleavage site), TEV protease, as well as EGFP and mKATE expressing plasmids, were introduced into HEK-FT cells through transfection. EGFP serves as a reporter, while mKATE acts as a marker for transfection efficiency. Each plasmid was used at a concentration of 60 ng, with the total DNA amount adjusted to 400 ng using an empty plasmid. Transfection was carried out using a fast-forward protocol, where cell plating and transfection occurred on the same day.

As a control, a wild-type MS2-cNOT7 (WT) lacking any cleavage site was included. Flow cytometry analysis (FACS) was conducted 48 hours post-transfection to quantify EGFP expression levels. The

results revealed that the introduction of TEV protease led to cleavage of the specific cleavage site, causing the separation of MS2-cNOT7. The dot plot in Figure 1 illustrates the outcome of TEV protease transfection.
Both the wild-type and engineered (with cleavage site inserted) MS2-cNOT7 exhibited good repression of EGFPx8MS2. However, upon the addition of TEV protease, EGFP expression significantly increased. Transfection with WT MS2-cNOT7 along with TEV protease (WT+TEVp) demonstrated a nonspecific increase in EGFP expression.

Table 1: Transfection conditions for testing TEV protease activity

Condition	Components
Negative control	EGFP+ mKate
TCS	MS2-TCS-CNOT7 + EGFP+ mKate
TCS+TEVp	MS2-TCS-CNOT+TEVP + EGFP+ mKate
WT	MS2-CNOT7 + EGFP+ mKate
WT+TEVp	MS2-CNOT7+ TEVP+ EGFP+ mKate
UN	Un-transfected cells
UN+A	Un-transfected cells + Transfection reagent

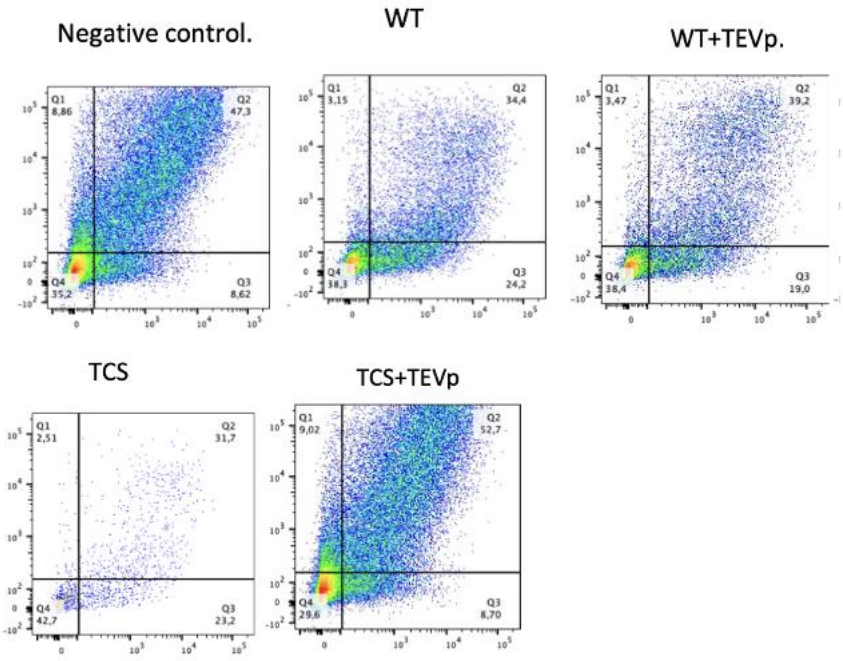


Figure 1: TEV protein transfection dot plots. The x-axis represents the EGFP expression level while the y-axis represents the mKATE expression. mKate transfected to all conditions as a transfection control. EGFP and mKATE are present in all samples unless stated otherwise. Negative control shows a baseline of EGFP activity since it is only transfected with EGFP and mKATE. WT samples have wildtype MS2-CNOT7 which is repressing the EGFP translation. WT+TEVp has TEV protease transfected with wild-type MS2-CNOT7. TCS samples include MS2-TCS-CNOT7 (MS2-CNOT7 with TEV cleavage sequence). TCS+TEVp samples have TEV protease transfected with MS2-TCS-CNOT7. TCS+TEVp samples are showing the rescue of EGFP translation by cleaving the cleavage site between MS2-CNOT7

Figure 2: Graphical representation of EGFP and mKATE expression of TEV protease transfection. Data in the left barplot represent the geometric mean and standard deviation of the means of EGFP. Data in the right barplot represents the geometric mean and standard deviation of the means of mKATE. Values are calculated based on the Q 2(double positive) population. Data show that in the presence of TEV, EGFP expression is rescued, indicating that MS2-TCS-cNOT7 was correctly cleaved and made non-functional.

Based on the transfection data, the MS2-CNOT7 system demonstrates effective functionality in response to TEV protease. In summary, the negative control (NC) in which the EGFP is not repressed exhibits high EGFP levels. The addition of wild-type MS2-cNOT7 (WT) which is without a responsive cleavage site results in system repression, whereas adding TEV protease to WT MS2-CNOT7. Transfection leads to a slight increase of both EGFP and mKate fluorescence suggesting a non-specific effect that was already reported (Cella et al., 2018). Transfecting the cells with MS2-TCS-CNOT7 which is an MS2-CNOT7 system with the TEV protease cleavage site showed repression of the EGFP fluorescent level. Introducing the TEV protease along with MS2-TCS-CNOT7 transfection showed the rescue of EGFP fluorescent levels through cleaving the cleavage site. Through all control and experimental conditions, it is evident that TEV protease cleaves the cleavage site between MS2-TCS-cNOT7, rescuing the expression, and thereby enhancing EGFP translation. The details of the transfection conditions are outlined in Table 1.
Next, we tested the MS2-cNOT7 responsive to TVMV. TVMV is a virus that belongs to the same family as TEV, but with proteases orthogonal that recognize specific cleavage sequences. In experiments involving HEK-FT cells, all plasmids were transfected at a concentration of 60 ng, with the total DNA amount adjusted to 400 ng using pEMPTY. FACS analysis was conducted 48 hours post-transfection.

Table 2 Transfection conditions for testing TVMV protease activity

Condition	Components
Negative control	EGFP+ mKate
TVMV-CS	MS2-TVMV-CS-CNOT7 + EGFP+ mKate
TVMV-CS+TVMVp	MS2-TVMV-CS-CNOT+TVMVP + EGFP+ mKate
WT	MS2-CNOT7 + EGFP+ mKate
WT+TVMVp	MS2-CNOT7+ TVMVP+ EGFP+ mKate
UN	Un-transfected cells
UN+A	Un-transfected cells + Transfection reagent

In summary, the negative control (NC) in which the EGFP is not repressed exhibits high EGFP levels. The addition of wild-type MS2-CNOT7 (WT) which is without a responsive cleavage site results in system repression, whereas adding TVMV protease to WT MS2-CNOT7. ransfection shows a slight increase in EGFP levels as already observed and commented above. Transfecting the cells with MS2-TVMVCS-CNOT7 which is an MS2-CNOT7 system with the TVMV protease cleavage site showed repression of the EGFP
Similar to the action of TEV protease, TVMV protease demonstrated the ability to cleave specific cleavage sites, separating MS2-CNOT7 from each other and removing CNOT7 from the poly-A thereby facilitating EGFP translation.

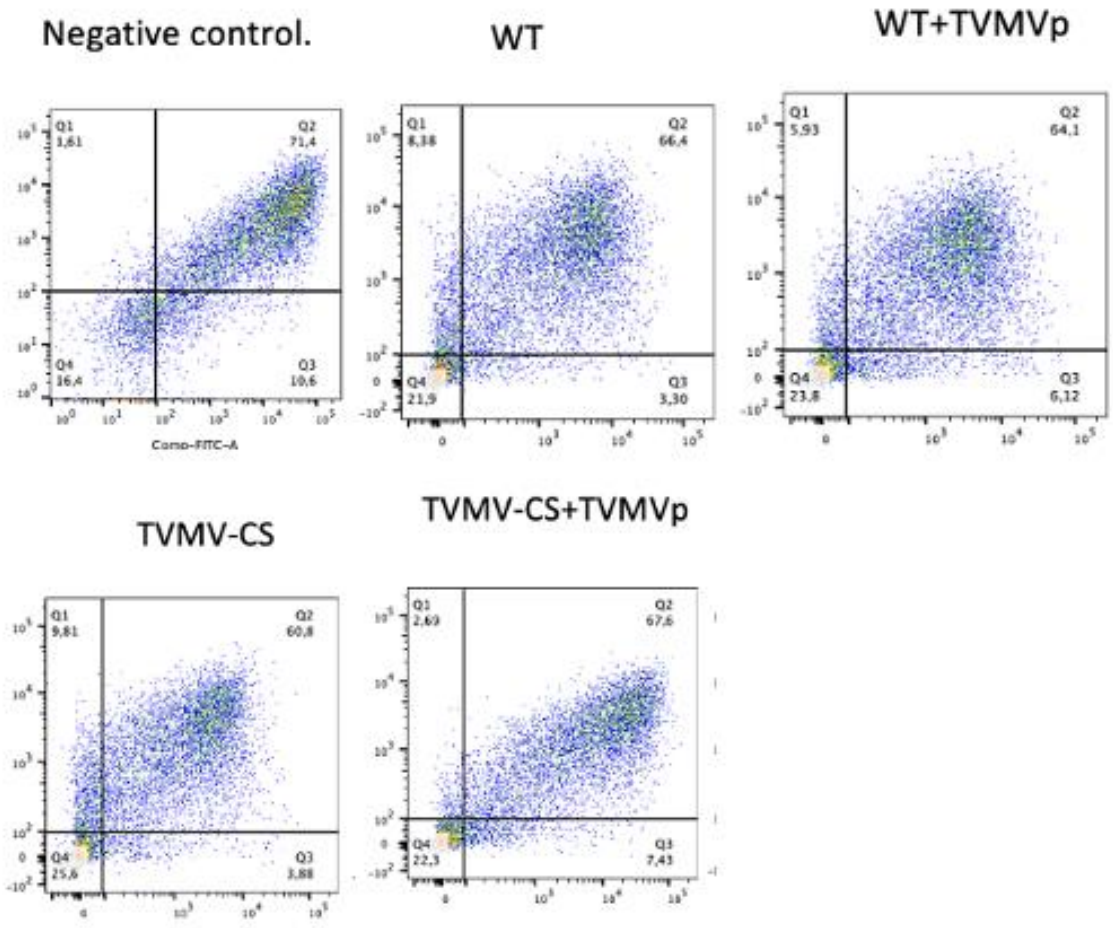


Figure 3: TVMV protein transfection dot plots. The x-axis represents the EGFP expression level while the y-axis represents the mKATE expression.mKate transfected to all conditions as a transfection control. . EGFP and mKATE are present in all samples unless stated otherwise. Negative control shows a baseline of EGFP activity since it is only transfected with EGFP and mKATE. WT samples have wildtype MS2-CNOT7 which is repressing the EGFP translation. WT+TVMVp has TV MVprotease transfected with wild-type MS2-CNOT7.T VMV-CS samples include MS2-TVMV-CS-CNOT7 (MS2-CNOT7 with TVMV cleavage sequence). TCS+TEVp samples have TVMV protease transfected with MS2-TVMV-CS-CNOT7. TVMV-CS+TVMVp samples are showing the rescue of EGFP translation by cleaving the cleavage site between MS2-CNOT7

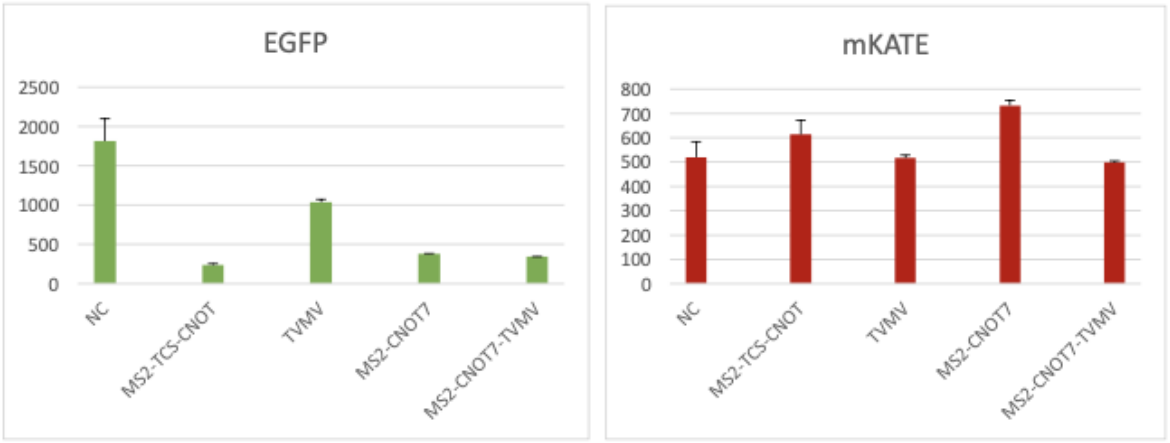


Figure 4: Graphical representation of EGFP and mKATE expression of TVMV protease transfection. Data in the left barplot represent the geometric mean and standard deviation of the means of EGFP. Data in the right barplot represents the geometric mean and standard deviation of the means of mKATE. Values are calculated based on the Q 2(double positive) population. Data show that in the presence of TVMV, EGFP expression is rescued, indicating that MS2-TVMV-CS-cNOT7 was correctly cleaved and made non-functional.

2.2 Testing Human-protease activity with MS2-CNOT7 system

MS2-cNOT7, a chimeric RNA-binding protein, has the capability of being manipulated to exhibit responsiveness towards a viral protease. To initiate this process, plasmids containing proteases from SARS-CoV-2, HIV, and Dengue virus have been procured for experimental evaluation. Additionally, specific cleavage site oligonucleotides corresponding to the protein sequences outlined in Table 3 have been obtained to facilitate testing of the system with the selected human-infective viruses.

Table 3: Virus and cleavage site belong to proteases currently in the test

Virus	Cleavage sequence
Dengue virus	KRRR--TAGV
	SKKR--SWPL
	NTRR--GTGN
HIV-1	RQAN--FLGK
	AW--LEAQ
SARS-CoV-2	ATVRLQ--AGNATE
	PHTVLQ--AVGACV

To test this system with human-infective virus proteases, the pL-R1 backbone which encodes MS2-CNOT7 wild-type is used to clone MS2-CNOT7 with cleavage sequences. Two restriction sites between MS2 and cNOT7 proteins were designed to be inserted (Figure 5). However, PCR amplification of MS2 ORF was not successful (Figure 6).

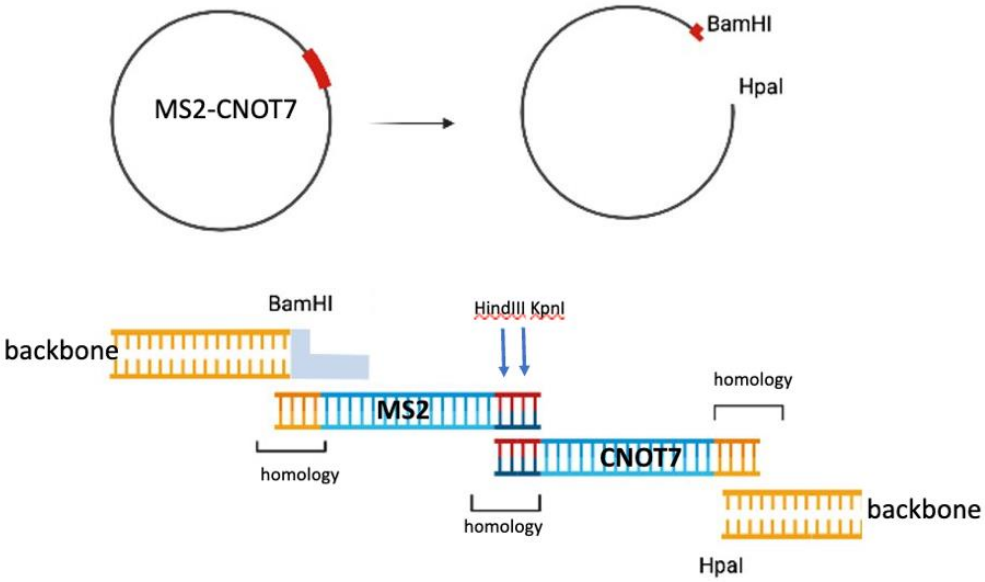


Figure 5 MS2-HindIII-KpnI-cNOT7 cloning strategy starting from MS2-CNOT (PL-R1) plasmid. The cloning is designed for In-Fusion cloning.

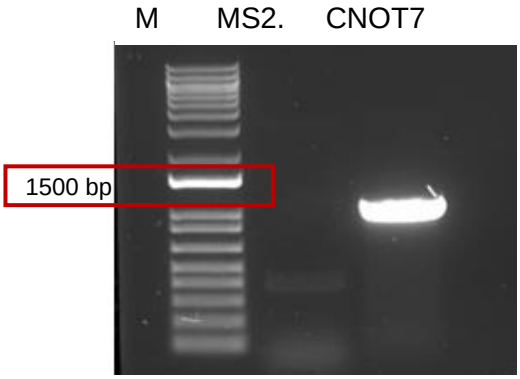


Figure 6: PCR of MS2 and CNOT7 ORFs for infusion cloning. PCR of the MS2 domain was not successful while CNOT7 amplified successfully.

Analytic digestion was performed on plasmid with BamHI and HpaI and analytical digestion results showed that BamHI was not able to digest the plasmid backbone. After obtaining this result, the PL-R1 plasmid is sequenced. Sequencing results showed that there is a 23 bp gap j upstream of the MS2 gene which was important for in-fusion cloning because 15 bp homologous sites were designed according to that sequence.

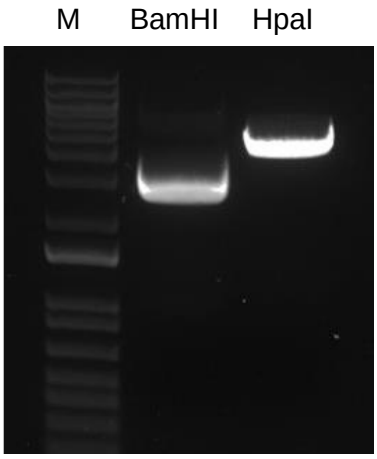


Figure 7: Single digestion of PL-R1 plasmid. M: marker, BamHI: Single digestion of PL-R1 plasmid with BamHI HpaI: Single digestion of PL-R1 plasmid with HpaI

Since the plasmid p152 (TVMV-CS plasmid) shares the identical MS2 sequence, MS2 ORF was efficiently amplified using p152 as a template. Subsequently, the p152 backbone underwent digestion with BamHI and HpaI, removing the MS2-CNOT7 segment. The amplified MS2 and cNOT7 fragments were then inserted into this backbone, generating an MS2-CNOT7 construct featuring the necessary restriction enzyme sites in between. Responsive cleavage sequences are inserted with oligo annealing. Newly generated construct verified by sequencing.

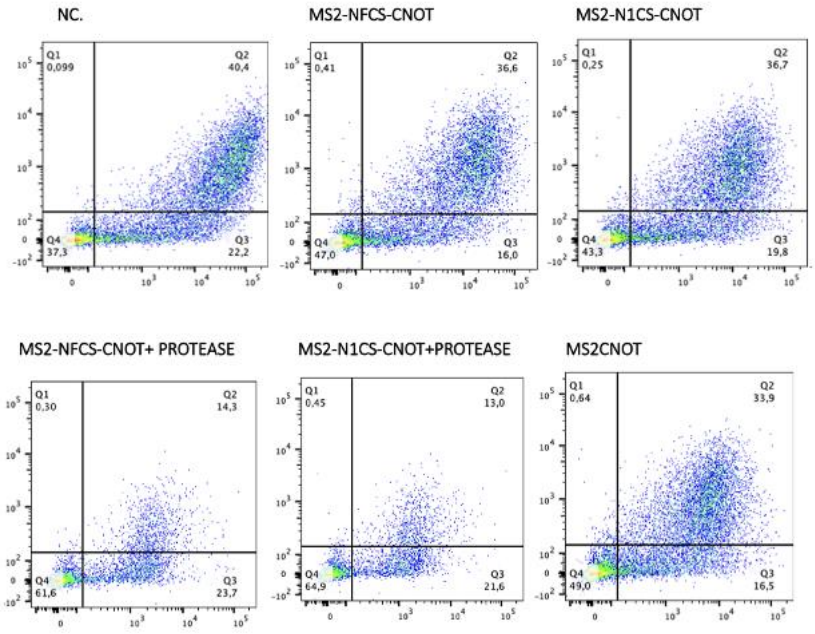
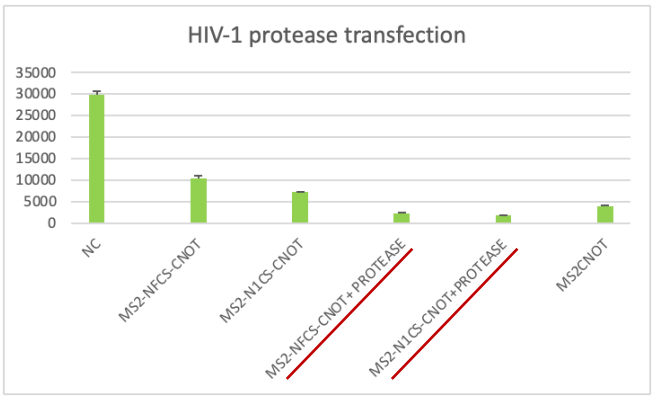


Figure 8: Testing HIV-1 protease activity with engineered MS2-CNOT7. The X-axis represents the EGFP expression level while the y-axis represents the mKATE expression. NC: negative control, NFCS: HIV-1 protease cleavage site 1, N1CS: HIV-1 protease cleavage site 2, Protease: HIV protease.

a.



b.

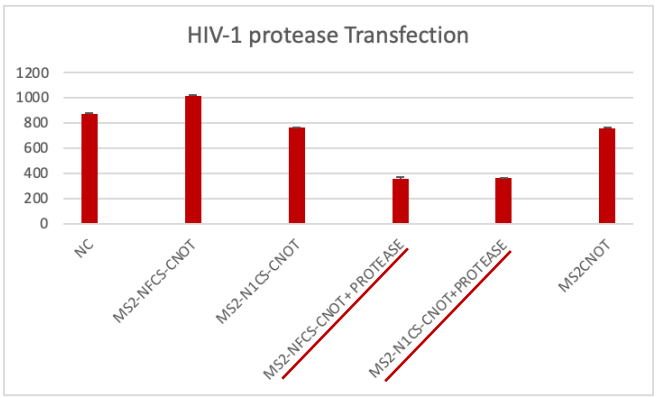


Figure 9: Graphical representation of EGFP and mKATE expression of HIV protease transfection. a Geometric meaning of the means of EGFP. EGFP expression values are calculated in the mKATE positive population. Standard error calculated based on replicates of two. b Geometric mean of mKate level of the transfection. Standard error calculated based on replicates of two

While transfection mixtures are prepared, the MS2-N1CS-cNOT7 and MS2-NFCS-CNOT7 plasmids were inadvertently omitted from the third and fourth samples as indicated in Figure 9 with red underlines. This oversight prevented the assessment of HIV protease activity with the responsive MS2-CNOT7 construct. However, the data revealed a significant decrease in EGFP expression even in the presence of HIV protease, suggesting its impact on gene suppression. Additionally, levels of mKATE decreased when the protease-encoding plasmid was introduced into the samples. This observation prompted speculation that the toxicity of HIV protease to the cells could be a contributing factor, considering its known ability to target internal proteins such as actin. Cell counts from FACS data were compared to investigate whether HIV protease influenced cell numbers. The initial HIV transfection results indicated a drastic decrease in cell count when the protease was co-transfected (Figure 10).

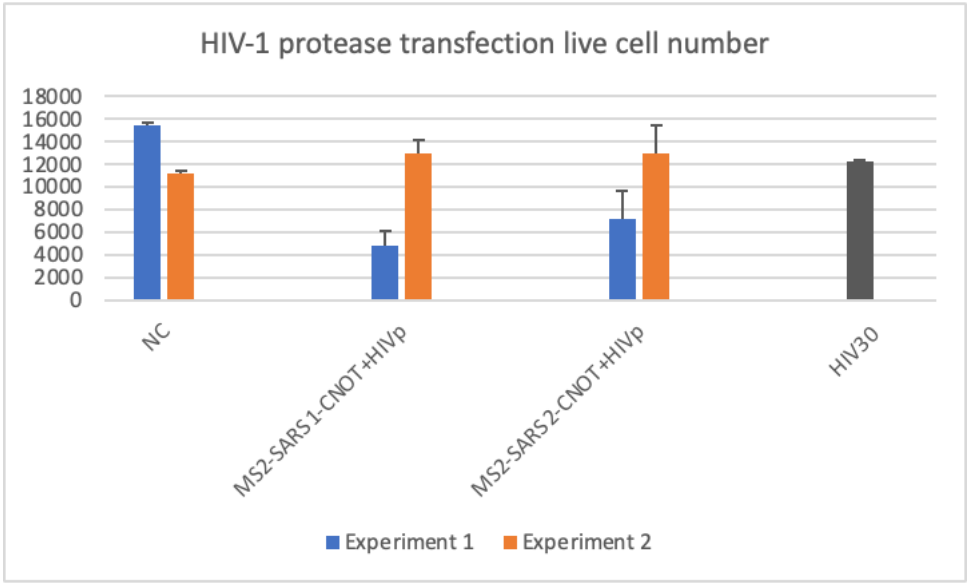


Figure 10: Graphical representation of live cell number of the transfection data. NC: Only EGFP and mKate plasmids are transfected. MS2-SARS1-CNOT7+HIVp: MS2-CNOT7 construct with SARS-CoV-2 protease cleavage site. MS2-SARS2-CNOT7+HIVp: MS2-CNOT7 construct with SARS-CoV-2 protease cleavage site. HIV30: EGFP, mKATE, and 30 ng of HIV protease. Experiments 1 and 2 represent two different experiments that were performed on different days. Grey block represents another experiment independent from the other two series.

Following these results, all the proteases are tested to confirm if they also have any toxicity to the cells. All proteases are co-transfected with EGFP and mKATE expressing plasmids to the HEK-FT cells (Figure 11). Since HIV protease is already known to affect cell number, the protease amount decreased by 50% and transfected by 30 ng instead of 60 ng.

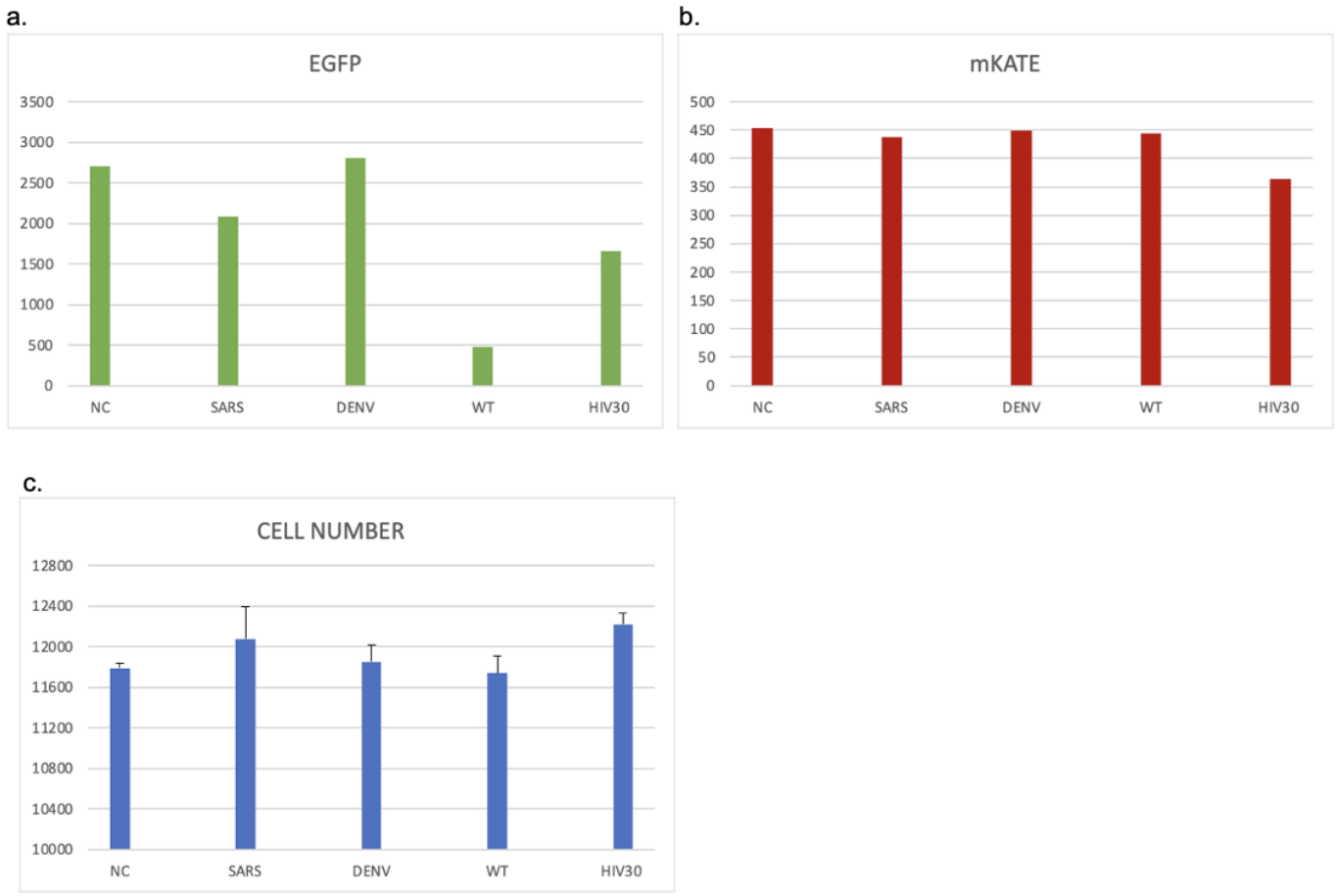


Figure 11: Transfection of human-infective proteases to HEK-FT cells. a Graphical representation of GFP level of the transfection b Graphical representation of GFP level of the transfection c Live cell number of the analyzed samples. NC: Negative control, only GFP and mKate, SARS: GFP + mKATE +SARS-CoV-2 protease, DENV: GFP + mKATE + Dengue protease, WT: GFP + mKATE +MS2-CNOT wild type, HIV30: GFP + mKATE + HIV-1 protease

Transfection results showed that SARS and Dengue viral proteases do not affect cell number. Following this result, SARS protease transfection has been designed with responsive cleavage sites (Figure 11). Transfection conditions are given in Table 4 as they are represented on Flow cytometry data (Figure 12).

Table 4: Transfection condition for testing SARS1 and SARS2 cleavage sites.

Condition	Components of the condition
NC	EGFP+mKate
MS2-SARS1-CNOT7	EGFP+mKate+MS2-CNOT7 with cleavage site number1
MS2-SARS1-CNOT+SARSp	EGFP+mKate+MS2-CNOT7 with cleavage site number+ SARS-Cov-2 protease

MS2-SARS1-CNOT+DENVp	EGFP+mKate+MS2-CNOT7 with cleavage site number+ Dengue protease
MS2-SARS1-CNOT+HIV-1p	EGFP+mKate+MS2-CNOT7 with cleavage site number+ HIV-1 protease
MS2-SARS2-CNOT+SARSp	EGFP+mKate+MS2-CNOT7 with cleavage site number+ SARS-Cov-2 protease
MS2-SARS2-CNOT+DENVp	EGFP+mKate+MS2-CNOT7 with cleavage site number+ Dengue protease
MS2-SARS2-CNOT+HIV-1p	EGFP+mKate+MS2-CNOT7 with cleavage site number+ HIV-1 protease

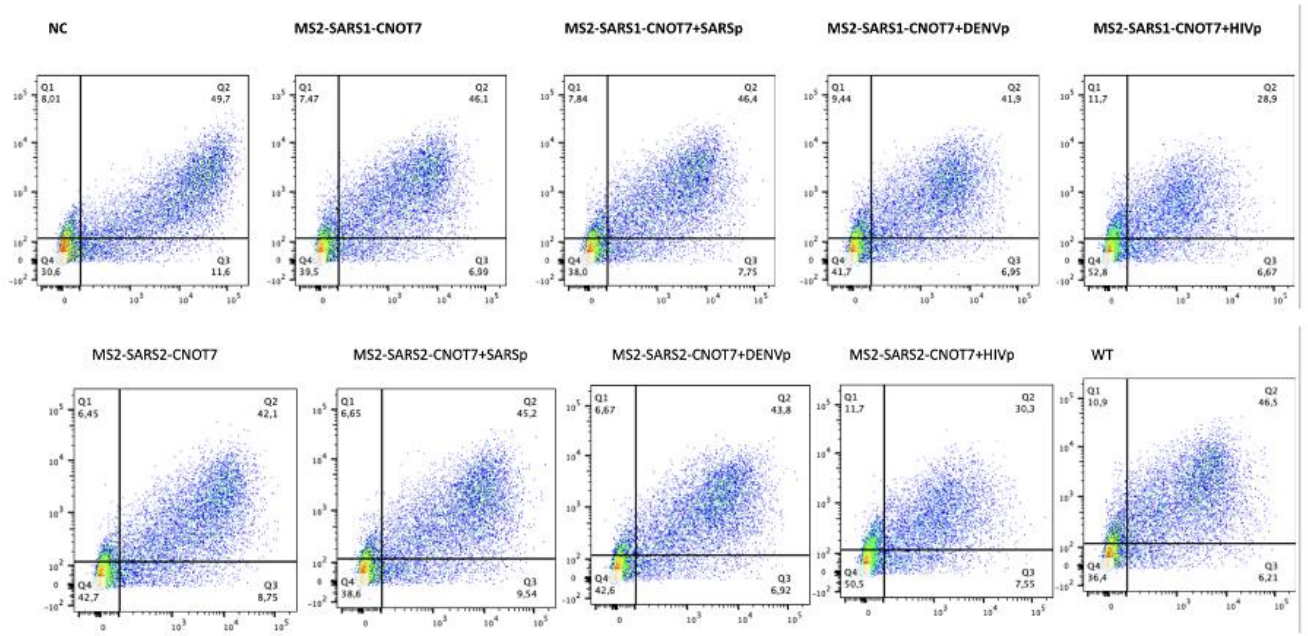


Figure 12: Flow cytometry analysis of transfection. NC: negative control transfected with only EGFP and mKate. EGFP and mKATE transfected in every condition. SARS1 and SARS2 represent cleavage sites. MS2-SARS1/SARS2-CNOT7 samples are transfected with MS2-CNOT7 with cleavage sequenceSARSp represents sars protease. DENVp represents dengue virus protease and HIVp represents HIV-1 protease. They are added to transfection according to the labeling of samples. WT samples have MS2-CNOT7 without any cleavage site. The X-axis represents the EGFP expression level while the Y-axis represents the mKATE expression.

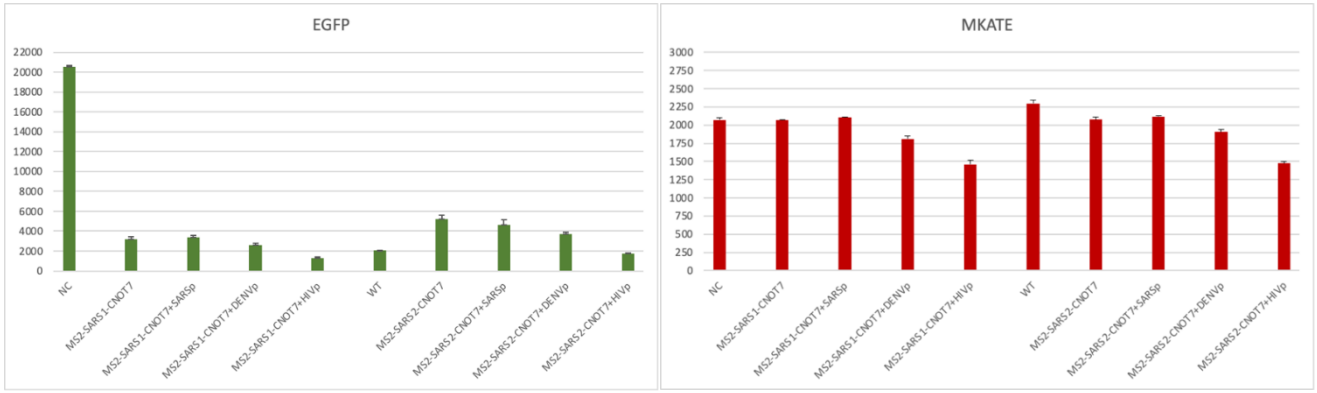


Figure 13 Graphical representation of EGFP and mKATE expression of SARS protease transfection. Data in the left bar plot represent the geometric mean and standard deviation of means of EGFP. Data in the right bar plot represent the geometric mean and standard deviation of the means of mKATE. EGFP expression values are calculated in the mKATE positive population

This experiment, however, showed that SARS protease is unable to cleave responsive cleavage sites. Transfection was repeated with SARS1 and 2 cleavage sites, and the results similarly showed that the SARS protease was either dysfunctional or not trasnfected efficiently. As a result of the literature review, it was seen that proteases are used 10 times less than in other studies (Resnick et al., 2021). Thereupon, SARS protease titration was performed, and its activity was tested between 60 and 400 ng (It was stated that 500 ng was also toxic and very few cells survived). Titration results again showed that SARS protease does not show any activity (Figure 14).

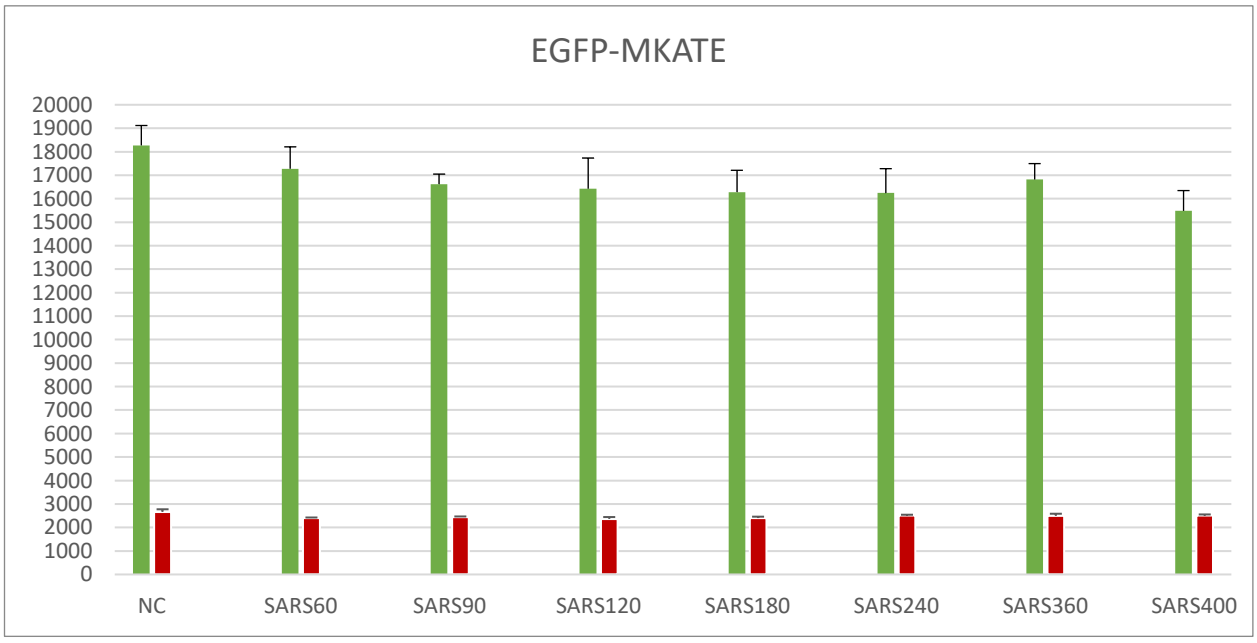


Figure 14 Graphical representation of EGFP and mKATE expression. Data represent geometric mean and standard deviation of means of EGFP

Based on these data, we are collaborating with a laboratory that is able to infect the cells with a virus. In the collaboration laboratory, cells will be directly infected with the SARS-CoV-2 virus. MS2-CNOT7 system will be tested on infected cells that naturally encode the protease.

3 Cell-Free Experiments

These experiments aim to see if a human-infective viral protease-responsive MS2-cNOT system works in a cell-free setup. In this experimental setup, the T7 MEGAscript kit was used to produce RNA output, and starting templates are always PCR products. Rabbit reticulocyte lysate (RRL) was used to produce protein output. Since the most important system in this part of the project relies on RNA-binding protein, the crucial step is the binding of a protein to the RNA structure. One of the disadvantages of this system was the colour of the lysate. Since it is produced from rabbit reticulocyte cells, which are slightly immature red blood cells, our reporter EGFP had to be changed to luciferase because of possible green-red interference. The output of this experiment will ultimately be measured in terms of the relative light unit. To develop the signal, a luciferase reagent will be introduced to the samples. When luciferase is present in the environment, D-luciferin in the luciferase reagent converts to oxyluciferin. This reaction produces oxyluciferin and light, that can be detected with a luminometer (Glomax) in terms of relative light unit (RLU). Luciferase will be translated in this experiment if viral protease can cleave the cleavage site between MS2 and cNOT7 proteins and release this chimeric protein from the MS2 loops. To test human-infective viral proteases, the MS2-cNOT system, reporter, and viral proteases were cloned into the T7-polyA backbone. Following plasmids were cloned for cell-free assays, T7-MS2-CNOT7-polyA, T7-MS2-TEVCS-CNOT7-polyA, and T7-Luc-x8MS-polyA.

Before testing human-infective viral proteases in cell-free, distinct types of RNAs are in vitro transcribed and tested in RRL to decide what type of RNA is more efficiently translated. Briefly, capped, and uncapped RNAs were produced with either natural nucleotides or modified ones and translation was tested to see the most efficient one to translate with the RRL system. According to the results, capped and unmodified nucleotide usage gave the most efficient protein translation (Figure 15).

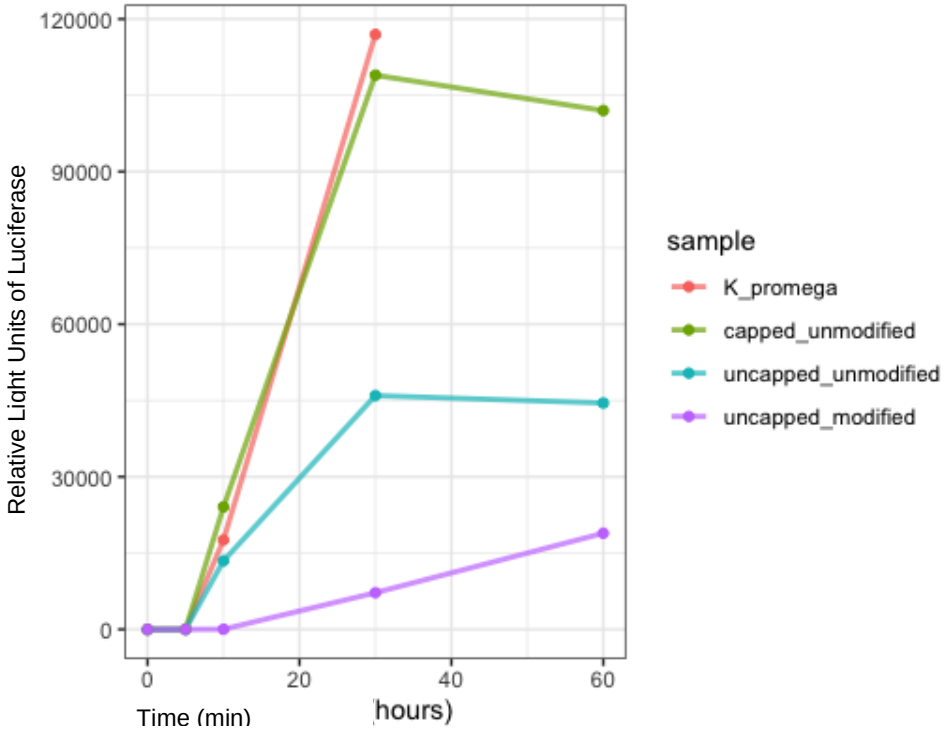


Figure 15: In vitro transcription of T7-Luciferase-polyA RNA. The red line represents Luciferase commercially available. The green line represents RNA produced with a cap and using unmodified nucleotides. The blue line represents RNA produced without a cap and using unmodified nucleotides. The purple line represents RNA produced without a cap and using modified nucleotides.

For in vitro translation, the Rabbit Reticulocyte Lysate system (RRL, Promega) is used. The RRL system consists of cytoplasmic extract that maintains the translation capabilities of a eukaryotic cellular cytoplasm. The experiment consists of testing the ability of (1) MS2-cNOT protein to inhibit Luciferase8MS2 RNA expression and (2) the specific de-inhibition (=activation of expression) of Luciferase8MS2 RNA by a viral protease. For this experiment, the most key factors to consider in preliminary tests are the following:

1) When should MS2-cNOT RNA be added compared to LUC-MS2 RNA? MS2-cNOT protein should already be there as in a protein form when LUC-MS2 RNA is not yet translated.
2) Is LUC protein expression affected by TEVp?
3) How much TEVp should be added to the lysate?

- Experiment 1: Test whether MS2-cNOT is able to inhibit Luc expression. Conditions:
  - LUC-MS2 (t=0) + MS2-cNOT (t=0)
  - LUC-MS2 (t=5 min) + MS2-cNOT (t=0)
  - LUC-MS2 (t=0) + MS2-cNOT (t=5 min)

- Experiment 2: Test whether LUC protein expression is affected by TEVp. Samples:
  - Luc-MS2 RNA (control)
  - Luc-MS2 RNA + TEVp concentration 1 (higher)
  - Luc-MS2 RNA + TEVp concentration 2 (intermediate)
  - Luc-MS2 RNA + TEVp concentration 3 (lower)

3.1.1 Testing MS2-CNOT and RNA interaction in the cell-free system

The brief logic of the MS2-CNOT chimeric protein is the MS2 protein binds to MS2 loops and brings the CNOT7 protein in close proximity to the poly-A tail. CNOT7 starts deadenylating and disrupting the mRNA stability causing the degradation of mRNA.
Since this system requires mRNA and protein at the same time, different addition times are tested. Since MS2-CNOT7 should be a protein form, it is always added to the system at the beginning of the reaction (time 0). Additionally, two different time points have been tested for the addition of luciferase with MS2 loops.
Cell-free systems include simply all the necessary machinery for transcription or translation. But unlike the cell-based systems resource is not ‘unlimited’. Since energy and other components have limited amounts in the cell-free reaction, translating different numbers of RNAs will create a bias in terms of translation efficiency. Considering this, mutated MS2-CNOT7 protein is generated with a simple mutagenesis reaction according to literature information (Peabody, 1993). Briefly, K56 and K58 sites have been changed, and binding affinity to the MS2 loops is disrupted. Mutated MS2-CNOT7 RNA is used to control resource usage.
In the first experiment, there were three different conditions which were, only luciferase8MS2 (Only Luc RNA), luciferase8MS2 + wildtype MS2-CNOT7, and luciferase8MS2+ mutated MS2-CNOT7. These three different conditions were tested for different time points. For all time points, MS2-CNOT7 protein was added to the reaction at the beginning. For time:0, luciferase8MS2 was also added at the beginning. For time: 5, luciferase8MS2 was added after 5 minutes, and for time:10 it was added to the reaction after 10 minutes. For the first experiment, each RNA was used as 70 fmol.

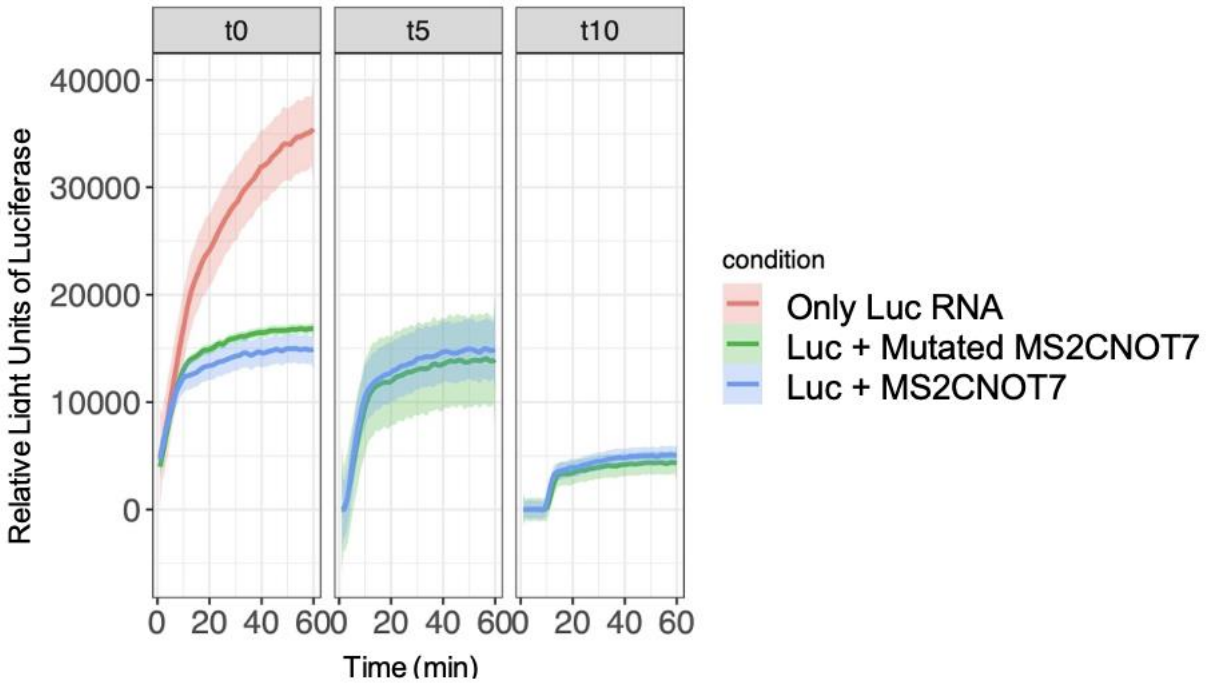


Figure 16: In vitro translation of MS2-CNOT7 chimeric protein with Luciferase8M2. The red line represents the data when only Luciferase8MS2 was translated. The green line represents the data when Luciferase8MS2 was translated with mutated MS2-CNOT7 RNA. The blue line represents the data when Luciferase8MS2 was translated with wild-type MS2-CNOT7 RNA. All RNAs were 70fmol.

The first experimental data showed that there is no difference between mutated and wild-type MS2-CNOT7 translation in terms of repressing the system. Mutated MS2-CNOT7 protein should not interact with luciferase RNA and only use the resources. In this scenario, the mutated MS2-CNOT7 condition should give the maximum value of luciferase translation when 2 different RNA is translated. However, wildtype MS2-CNOT7 was showing the same value meaning that either wild type is not causing any repression or mutated MS2-CNOT7 still interacting with luciferase (Figure 16).
The system is based on the repression of reporter luciferase. Since translating the system component in a 1:1 molar ratio (figure 16) did not give the expected repression, the repressor (MS2-CNOT7 protein) concentration is doubled, and the system is translated in a 1:2 translation with only two time points (Figure 17). The double MS2-CNOT7 experiment was repeated twice. Data showed that there is a difference between mutated and wild-type expression.

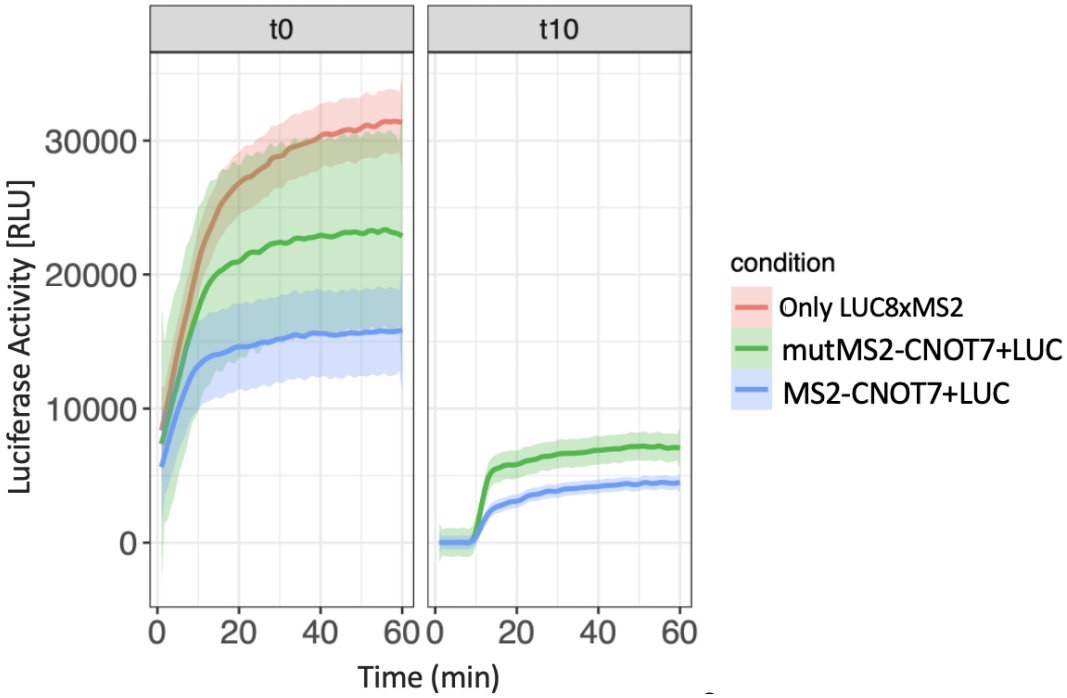


Figure 17: In vitro translation of MS2-CNOT7chimeric protein with Luciferase8MS2. The red line represents the data when only Luciferase8MS2 was translated. The green line represents the data when Luciferase8MS2 was translated with mutated MS2-CNOT7 RNA. The blue line represents the data when Luciferase8MS2 was translated with wild-type MS2-CNOT7 RNA. Luciferase8MS2 was used RNAs were 70fmol. MS2-CNOT7 both (wildtype and mutated) used 140 fmol.

Doubling the MS2-CNOT7 system showed a promising difference at time point 10 then the system tested with increasing the MS2-CNOT7 concentration to 4-fold (Figure 18). For the 4-fold experiment, unrelated RNA was included as a control. Unrelated RNA only uses the resources in the reaction mixture and does not interact with the Luciferasex8MS2. First, this setup tested for time 0 to have an overview of the 4-fold MS2-CNOT7 usage. Since unrelated RNA is not able to interact with reporter luciferasex8MS2 RNA, it should give the maximum translation yield when 2 RNAs are translated in the system. But unrelated RNA is shorter than MS2-CNOT7 RNA, and this difference causes different resource usage but still, it should be comparable with the repression.
Utilizing MS2-CNOT7 at a fourfold higher concentration compared to luciferase8MS2 resulted in significant differences in the translation of luciferasex8MS2. This indicates that MS2-CNOT7 has an impact on the translation process of luciferasex8MS2. But biologically, as a designed biosensor, the difference is not showing as high as expected. The same experimental design was repeated for time point 10.

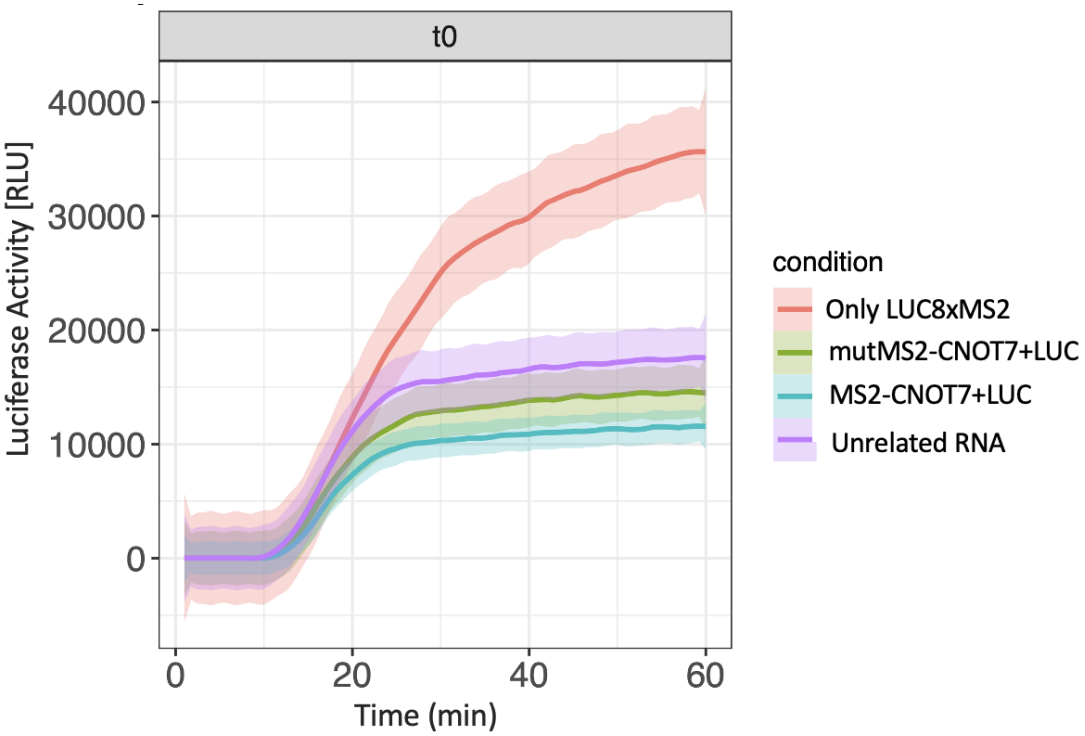


Figure 18: In vitro translation of MS2-CNOT7chimeric protein with Luciferase8MS2. The red line represents the data when only Luciferase8MS2 was translated. The green line represents the data when Luciferase8MS2 was translated with mutated MS2-CNOT7 RNA. The blue line represents the data when Luciferase8MS2 was translated with wild-type MS2-CNOT7 RNA. The purple line represents the data when Luciferase8MS2 was translated with unrelated RNA. Luciferase8MS2 was used RNAs were 70fmol. Both MS2-CNOT7 RNAs (wildtype and mutated) used 280 fmol. Unrelated RNA used the same nanograms as MS2-CNOT7

While testing the system with a 4-fold increase in MS2-CNOT7 for time point:10, an additional control was introduced into the experimental setup. Luciferasex8MS2 RNA was translated without a poly-A tail, to demonstrate the fate of RNA in the reaction without poly-A. The experimental results indicated that, unlike in cell-based systems, RNA can be translated into a protein independently of the presence of a poly-A tail (Figure 19).

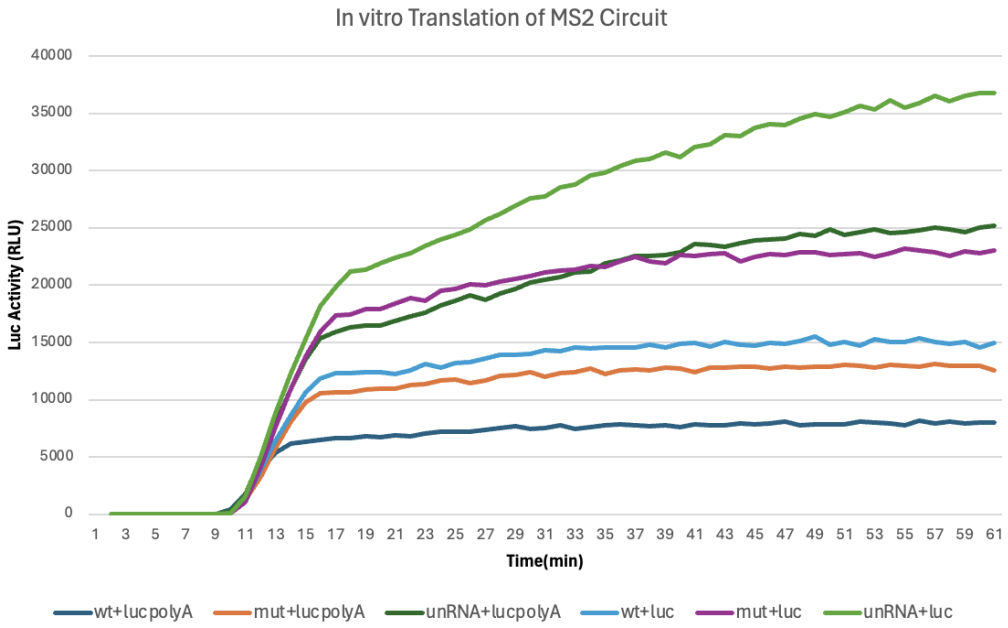


Figure 19: In vitro translation of MS2-CNOT7chimeric protein with Luciferase8MS2. Time:10

The availability of MS2-CNOT7 in the reaction influences translation, but this effect does not appear to be linked to the deadenylation of the template RNA. According to the literature data, RNA can be translated to protein with a high efficiency even without a polyA tail (Figure 20) (Michel et al., 2000).

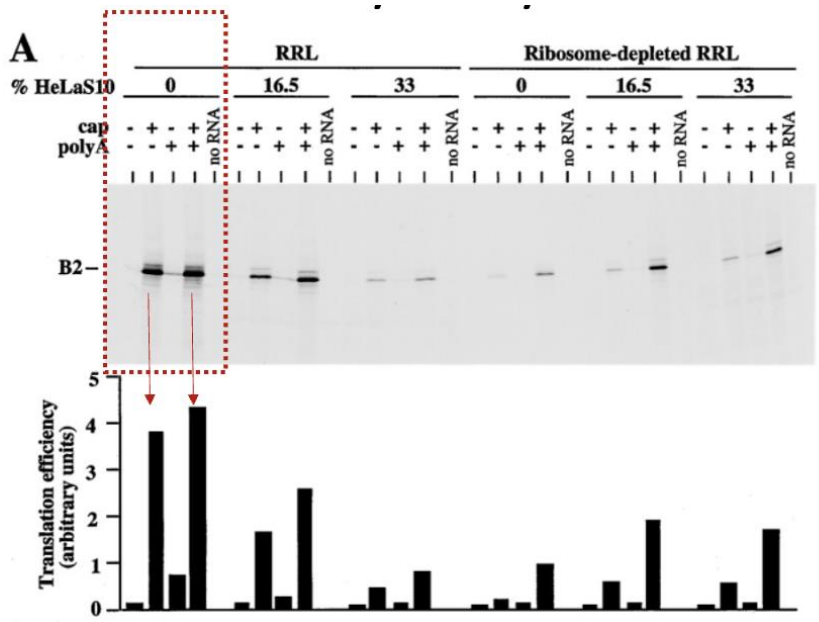


Figure 20: Translation of several types of RNAs Adapted from (Michel et al., 2000)*. The red arrows highlight the RNAs produced with or without polyA*

4 Conclusion

The MS2-CNOT7 system is founded upon RNA deadenylation and the disruption of RNA stability. According to the data, this system can inhibit the translation of reporter RNA. Moreover, it has been demonstrated that in the presence of TEV and TVMV proteases, when the two proteins are separated from each other, the translation of the reporter is restored. Nonetheless, the kinetics of reactions in cell-free systems differ from those in cell-based systems. While cell-based systems involve a period for the degradation of deadenylated mRNA, the translation process occurs within only 60 minutes in cell-free systems. As a result, the MS2-CNOT7 system is considered not suitable as an RNA-binding protein for biosensor design in a cell-free system.

In forthcoming investigations, the reporter RNA will undergo strategic modifications to enhance its functionality. Specifically, two k-turn motifs will be inserted into 5' of the luciferase. Consequently, the reporter RNA will be engineered with a sequence denoted as 2xkturn-luciferase-polyA. To impart viral responsiveness, the repressor protein L7Ae, known for its RNA-binding capabilities, will be re-engineered. Cleavage sites responsive and specific to human-infective viral protease will be inserted into L7Ae. Since L7Ae has the ability to bind k-turn motifs, L7Ae will modulate the luciferase activity. Specific and responsive cleavage site insertion will ensure the L7Ae's viral protease specificity. This system will be tested and validated in a cell-free.

5 Bibliography

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