iVEPOP

in vitro eternal expression of protein

iGEM GIFU_TOKAI
1th November, 2019

Presented by
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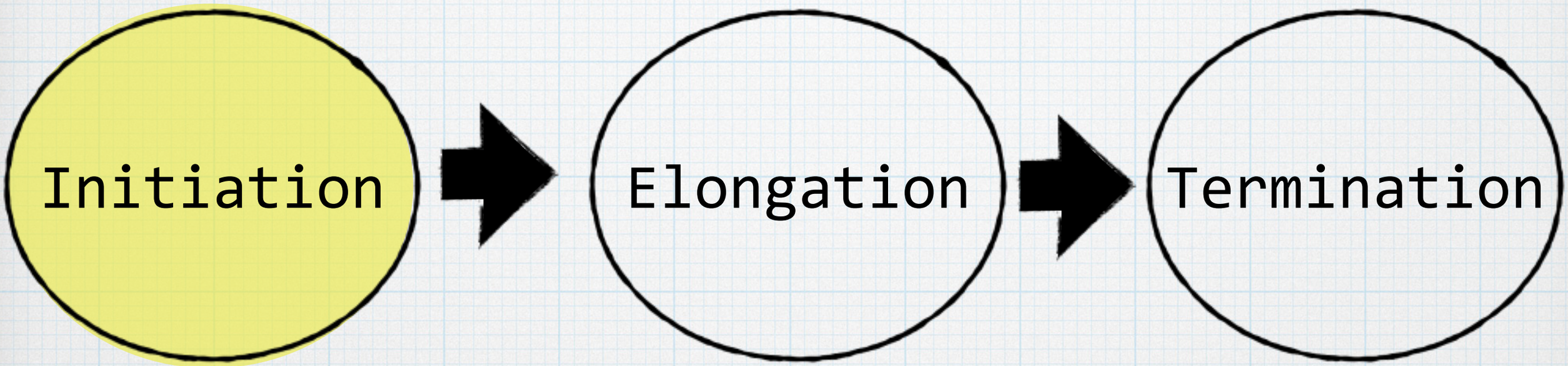
Inspiration

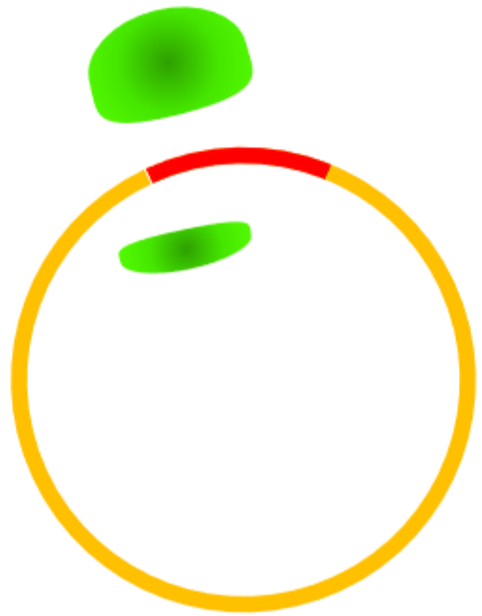
-Why do we start our project?-

The process of protein synthesis has three steps



If we can skip the rate-limiting step,
it is possible to synthesize proteins quickly.

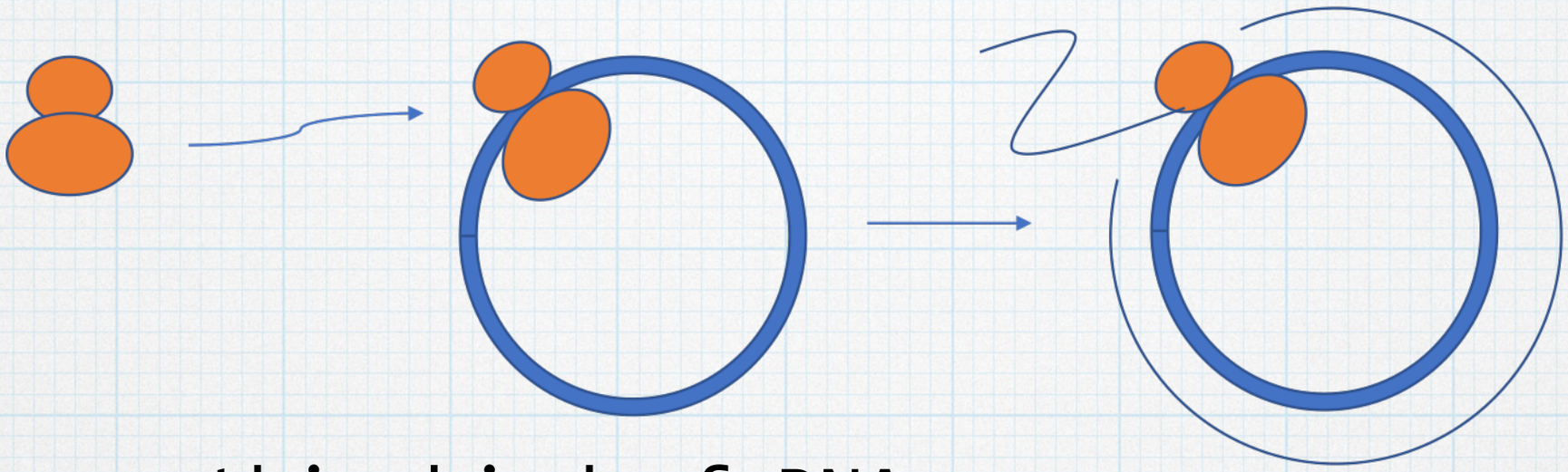
Naoko ABE at el. Rolling Circle Translation of Circular RNA



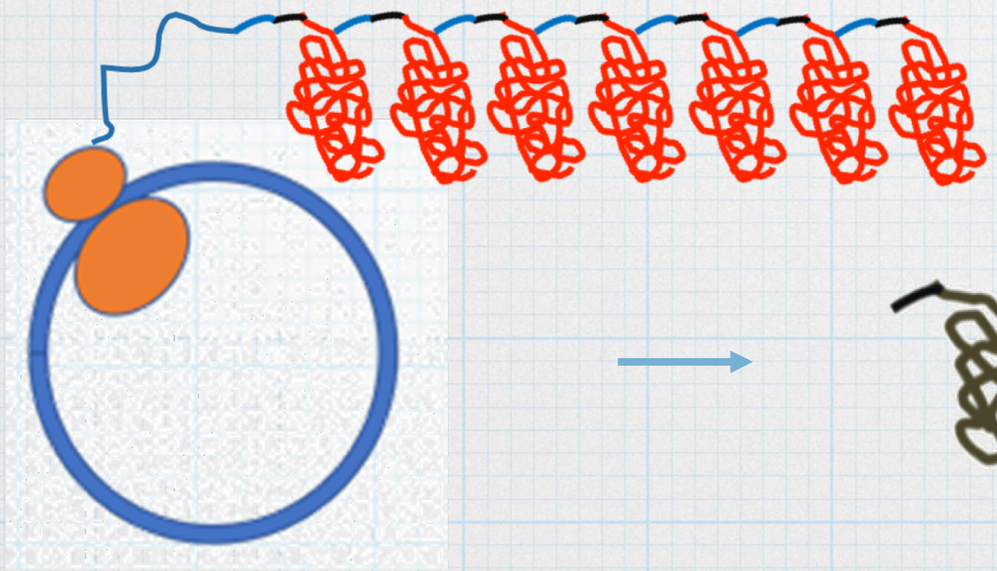
Background

-How do we make it possible?-

Background circular RNA



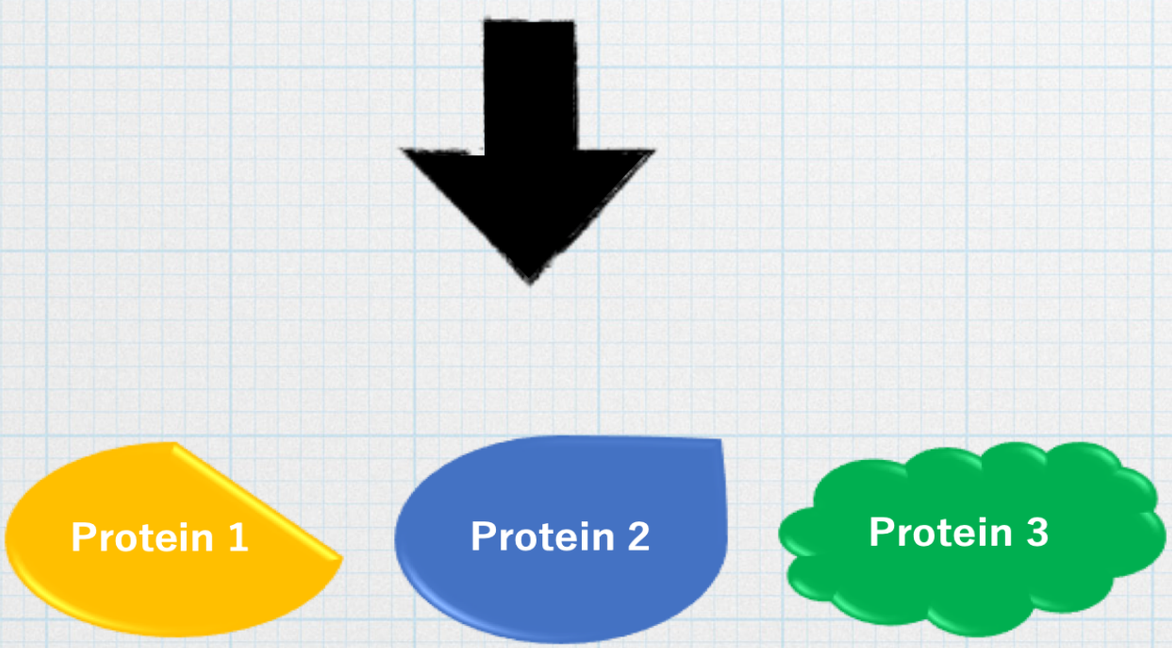
If we have this kind of RNA
which does not have stop codon,
What will happen?



It easily aggregates and
has no function.

Background translation coupling

Translation-coupling is a phenomenon which occurs in bacterial operon



Ribosome flows through stop codon at CDS 1 and CDS 2.

Several proteins are expressed from one mRNA. This phenomenon is called translation coupling.

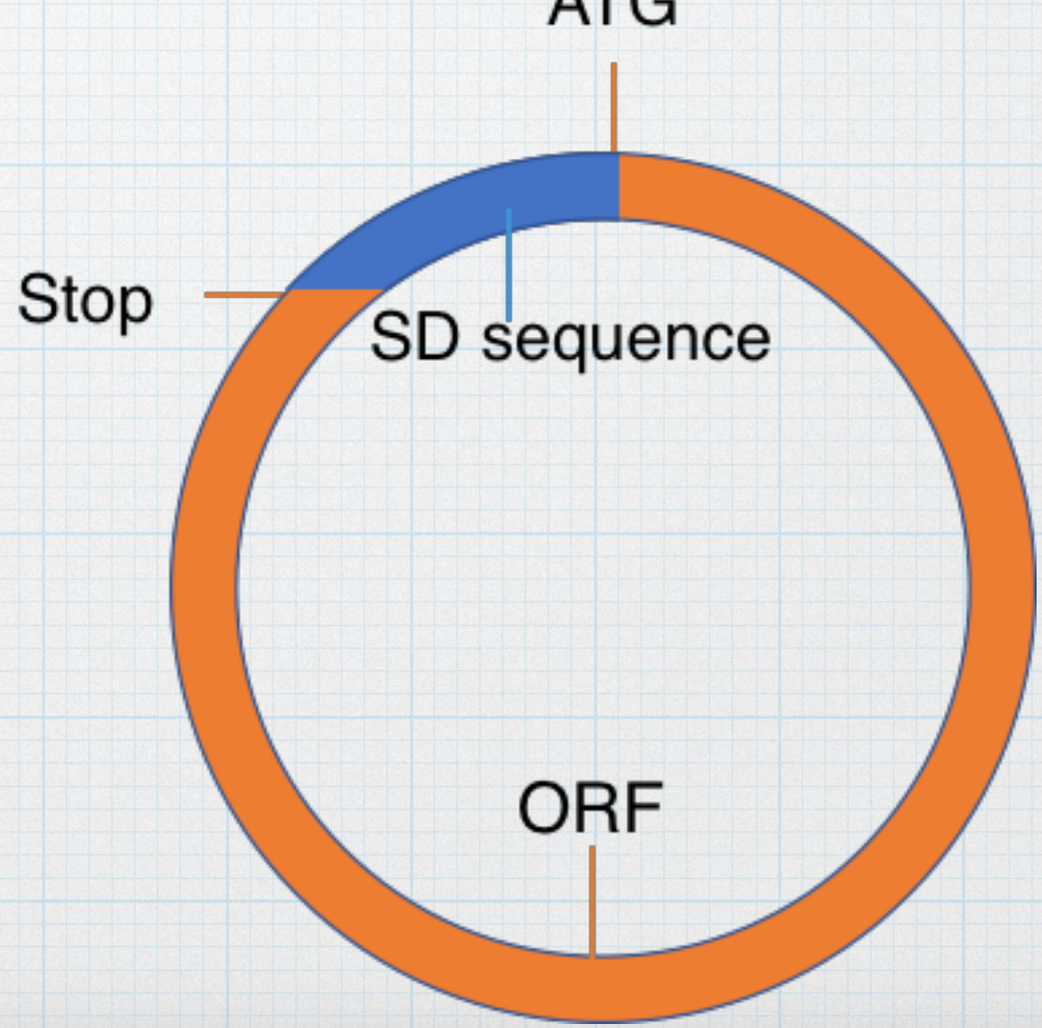
Our project

SD sequence

gggaaggagatatacca sfGFP t-3′

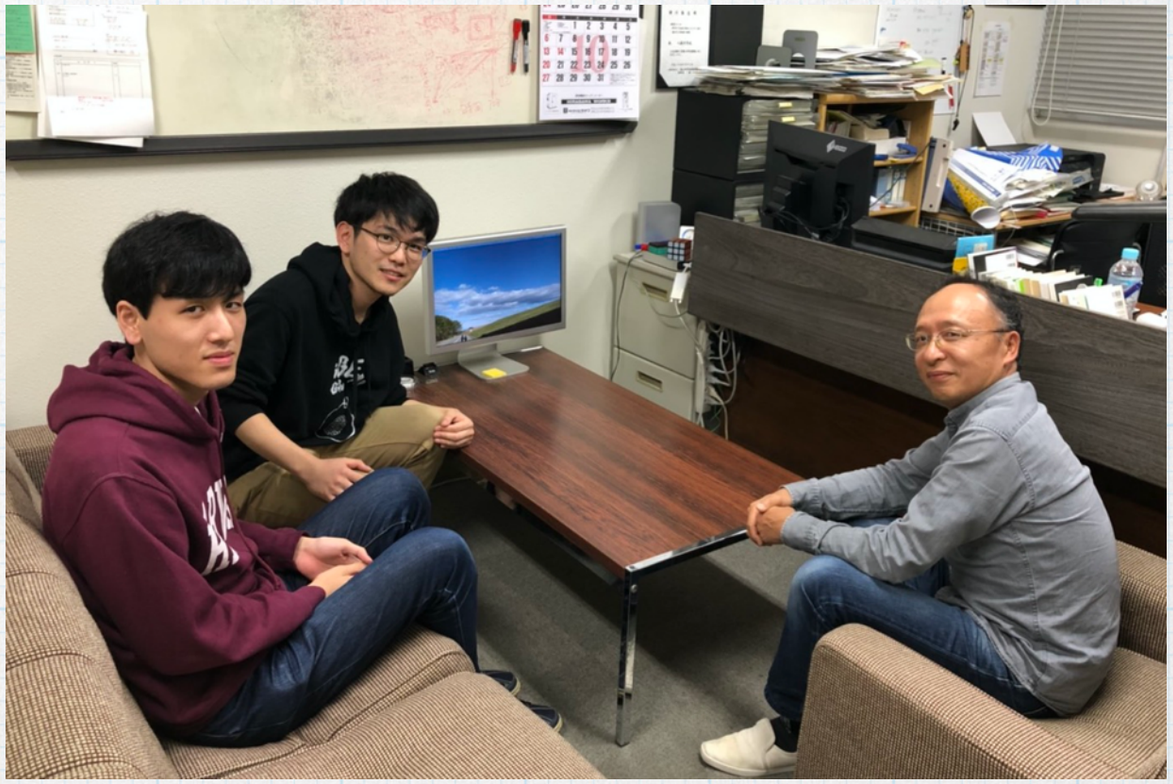
ATG

To generate functional proteins, We applied the translation-coupling system.



Background

Our knowledge were also provided by them



1.Getting suggestions from GeneFrontier

2. Consulting with Prof. Yokogawa

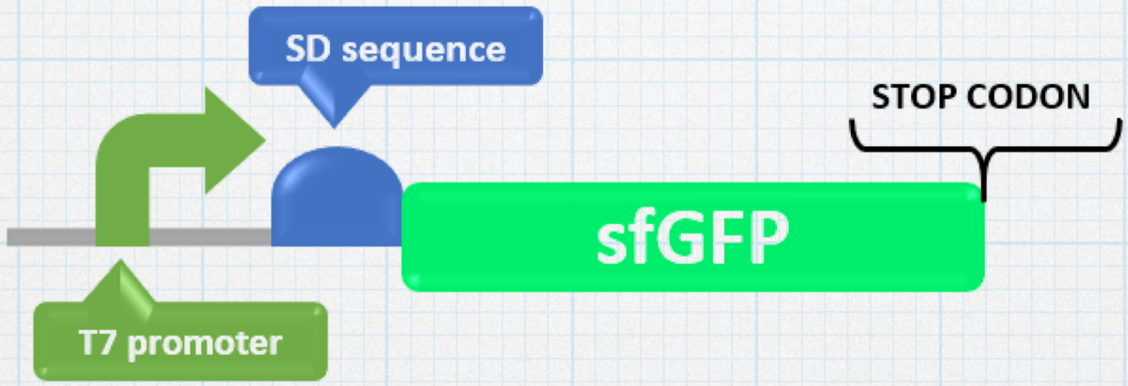
Method and the Result of our study

Method

1. The DNA template amplification
2. Transcription
3. mRNA circularization
4. Linear RNA digestion
5. Expression
6. The assay of proteins

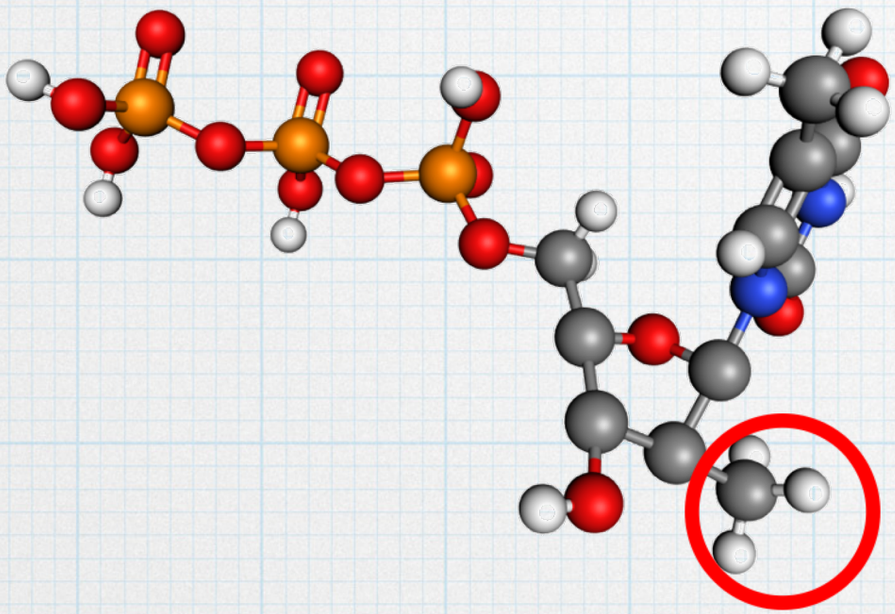
Project

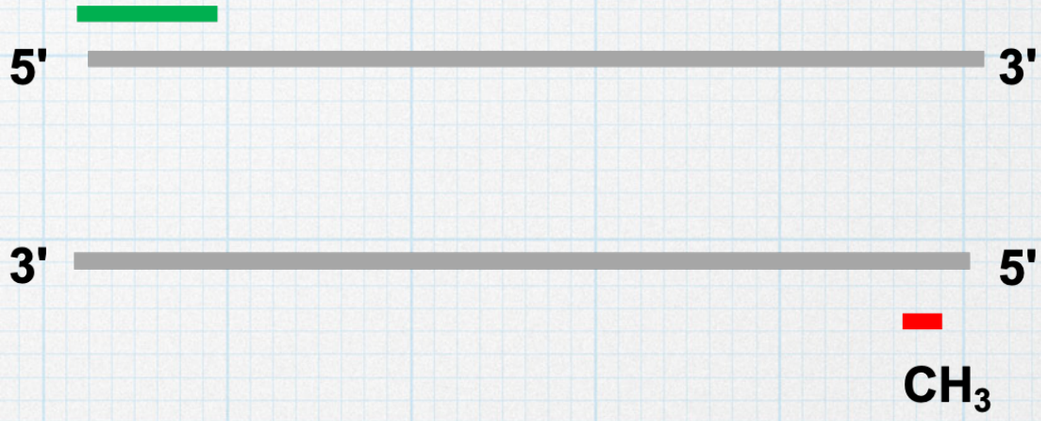
primer set	sequence
T7_Abe_Fw	cgcggatcctaatacgactcactatag
Rev_-o-methyl_primer_igem	atttgtacagttcatccat



- Generated DNA fragments contain T7 promoter, SD sequence, sGFP and spacer.
- The spacer is derived from translation coupling as a operon.

Project

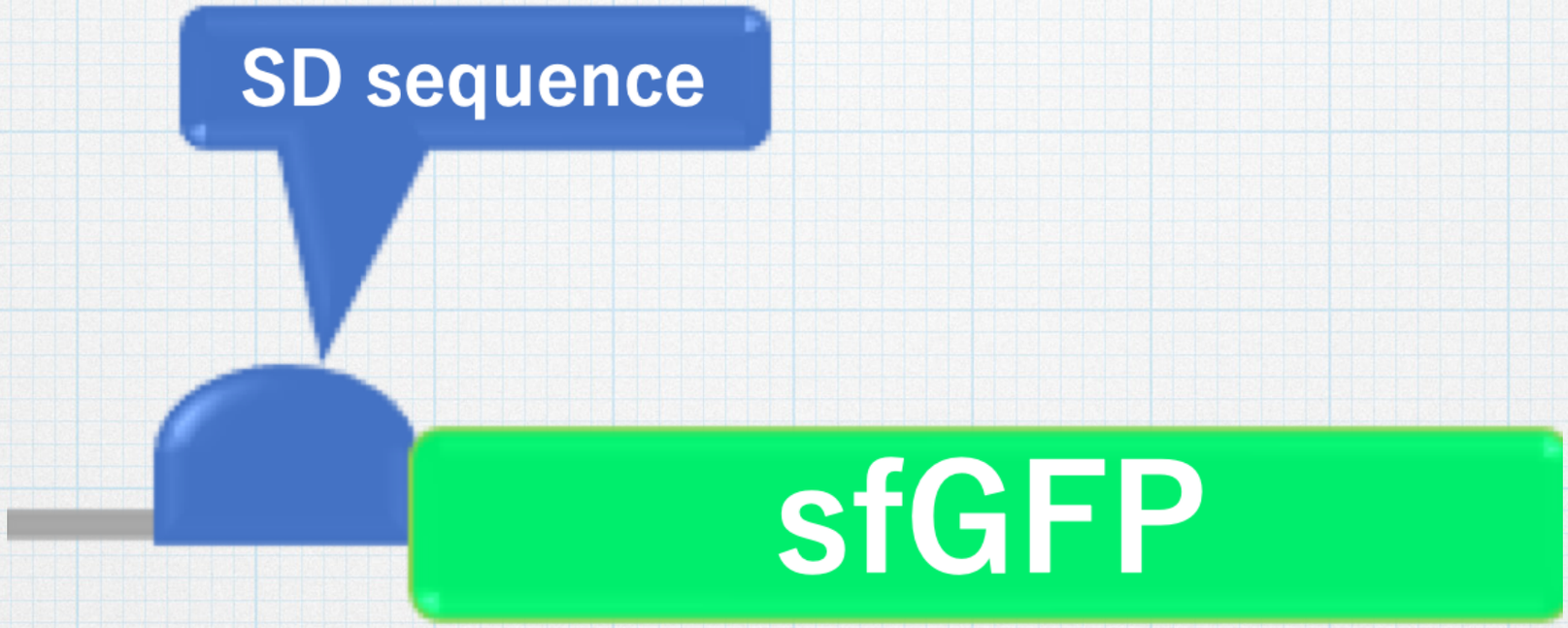




We changed the Second base(T)of the reverse primer into 2-o-methyl RNA to prevent random addition of the base at the end of 3’.

Project

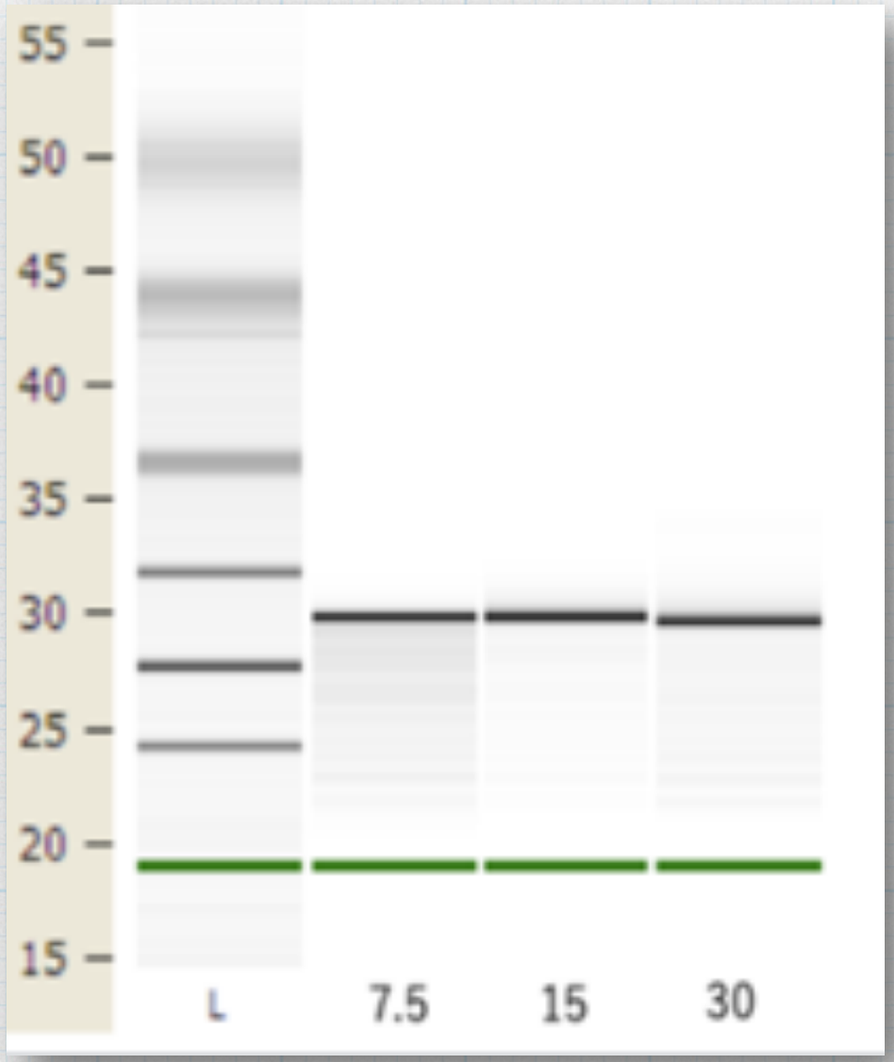
mRNA



The DNA which has T7 promoter is specifically recognized and transcript by T7 RNA polymerase.

→ Add Guanosine monophosphate(GMP).

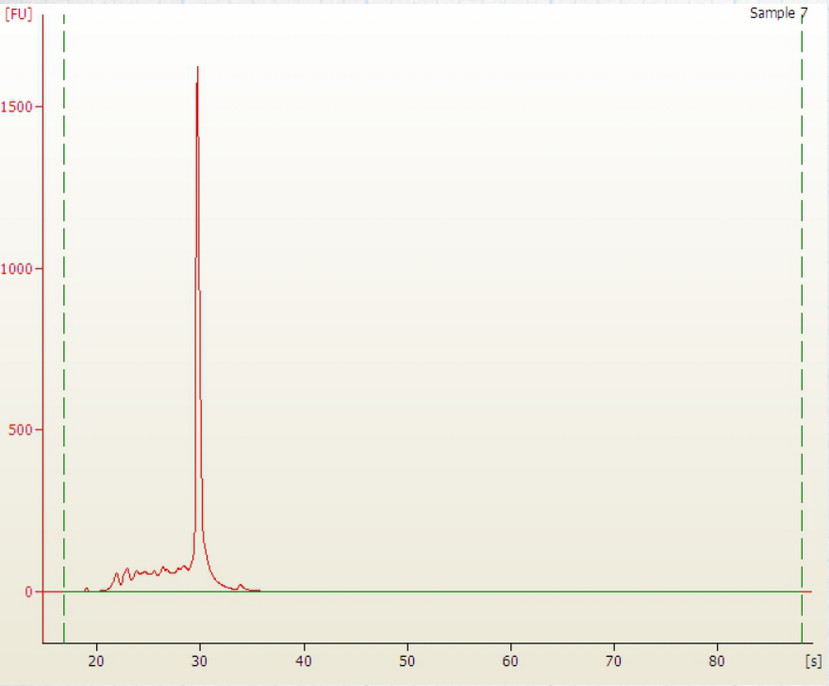
Result (transcription of linear RNA added GMP)



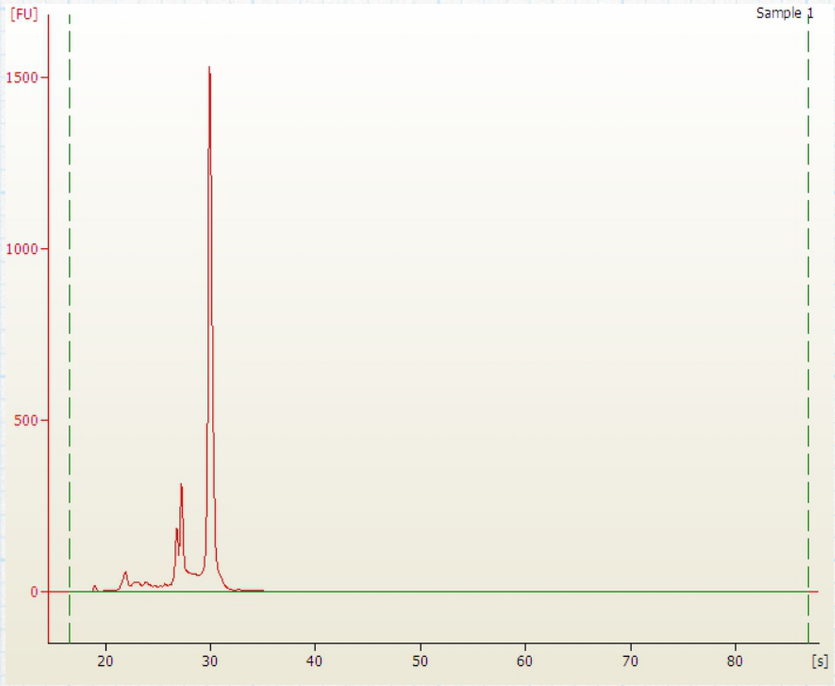
Amount	Component
7.5, 15,30 mM	GMP
2 uL	ATP
2 uL	CTP
2 uL	GTP
2 uL	UTP
2 uL	10×Reaction Buffer
0.1 ug	linear template DNA
2 uL	Enzyme Mix

Line.2 7.5 mM,
Line.3 15 mM, Line.4 30 mM
added GMP to MEGAscript

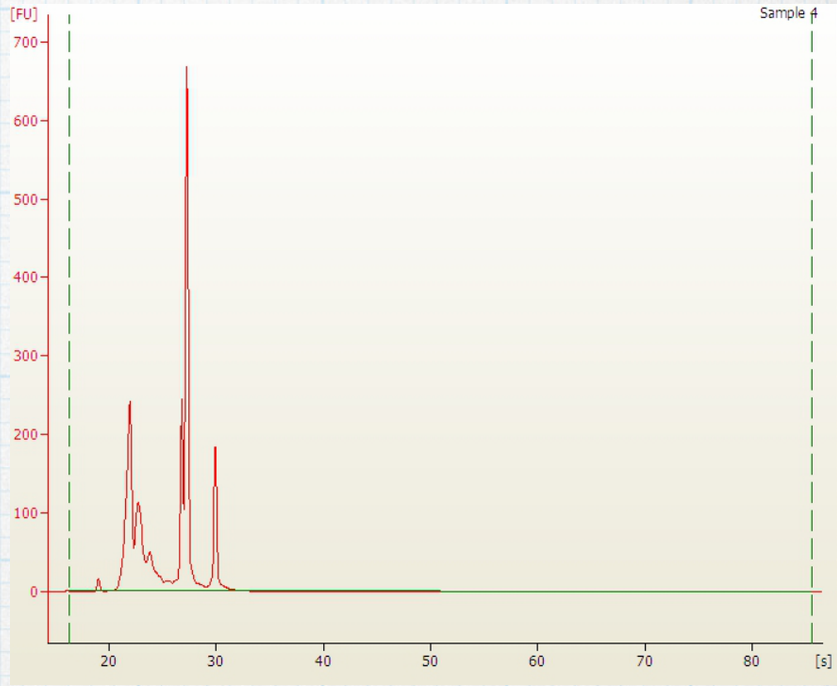
Project



GGG



GG

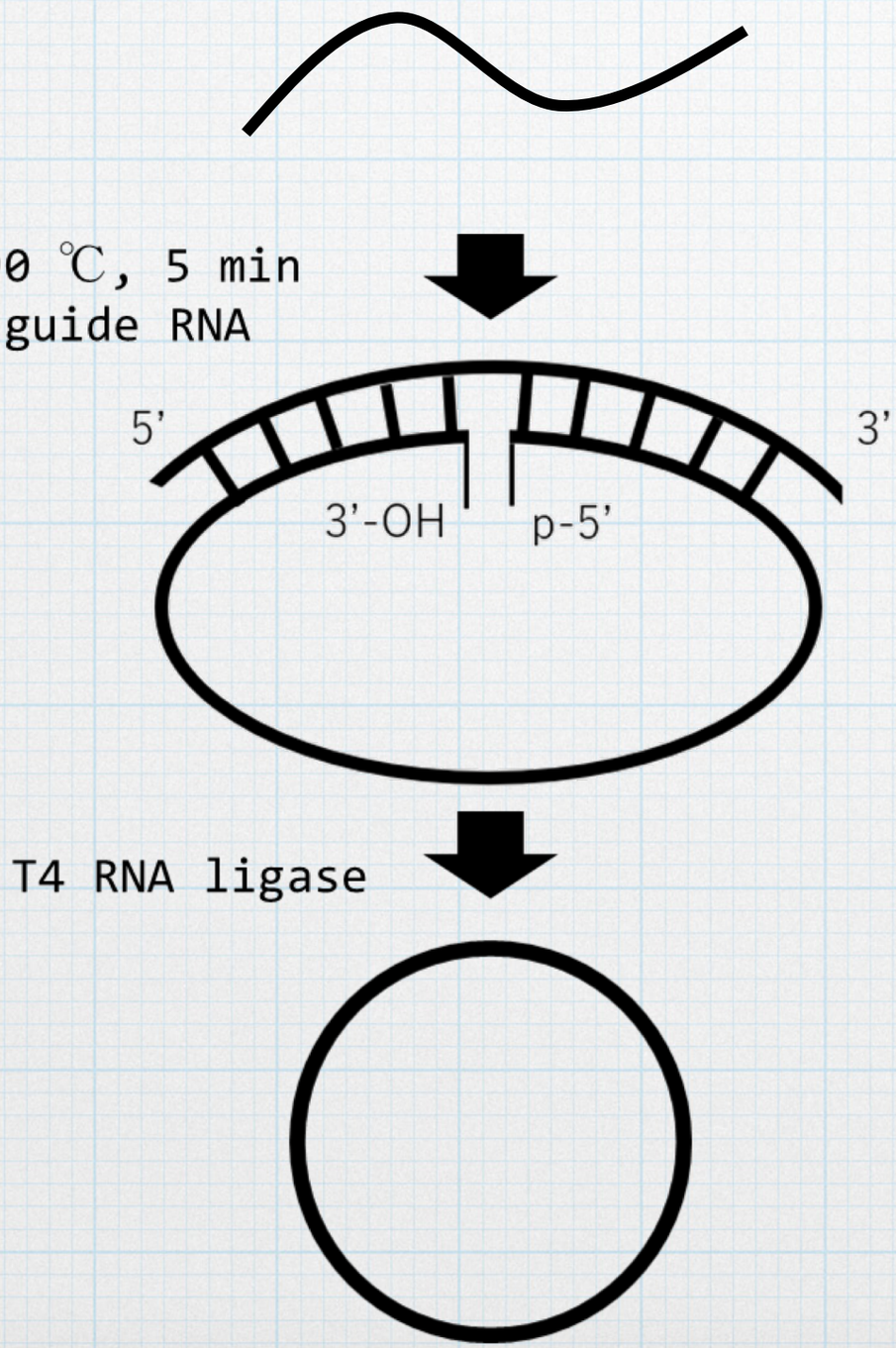


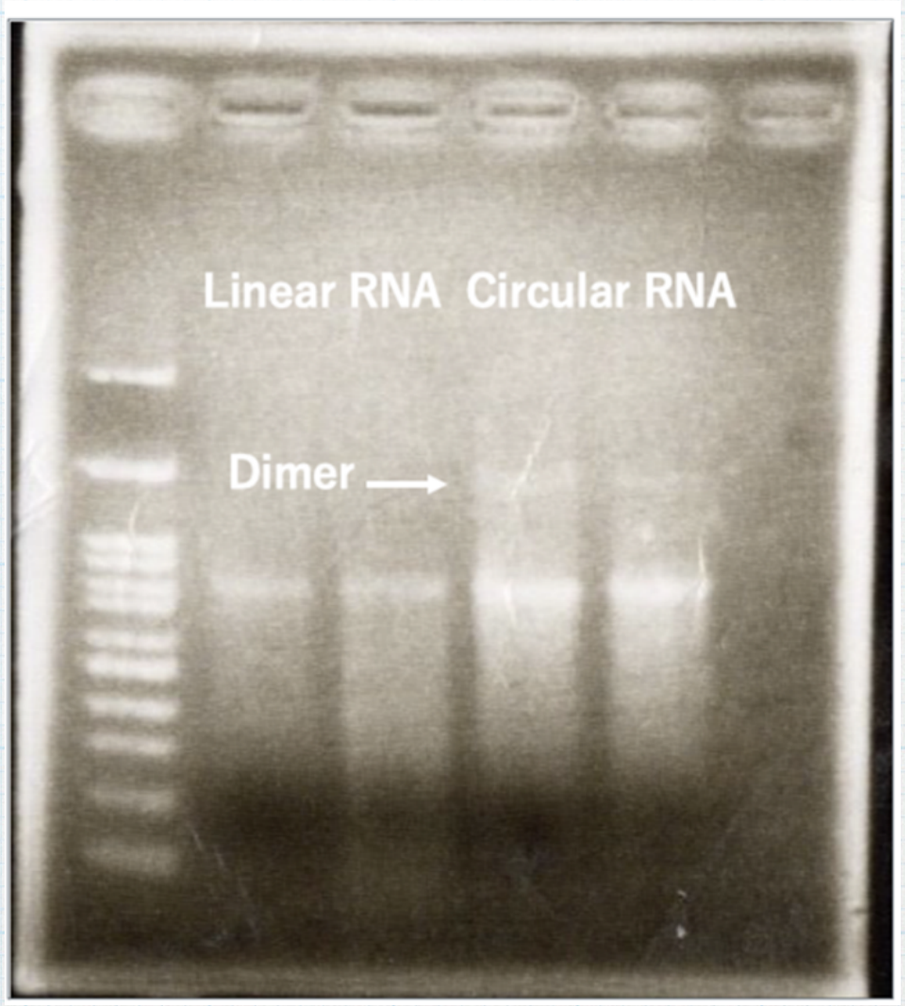
G

T7 promoter

taatacgactcactataGGGaaggag

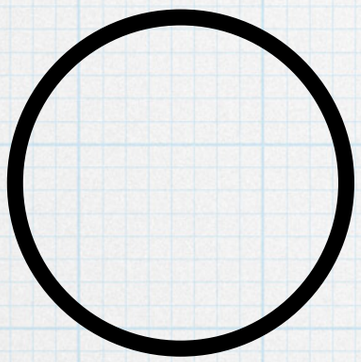
Project



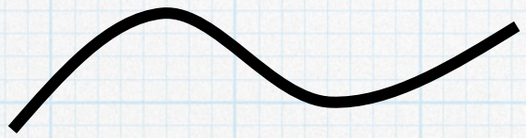


Line3 0.5 μM, Line4 0.2 μM (circular)

Project

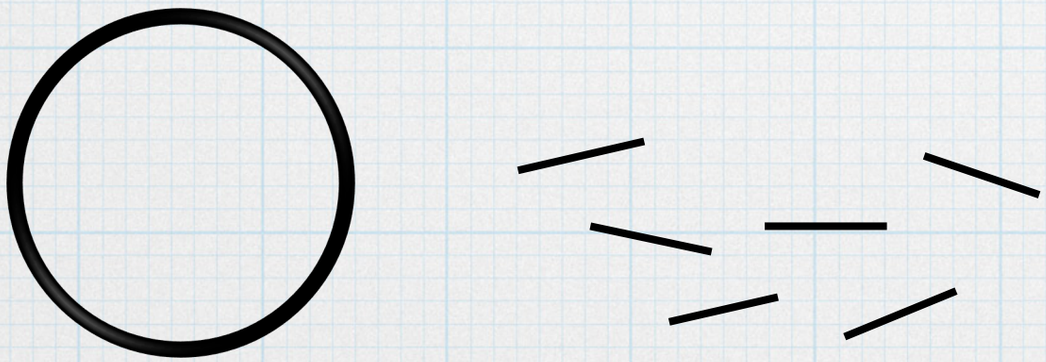


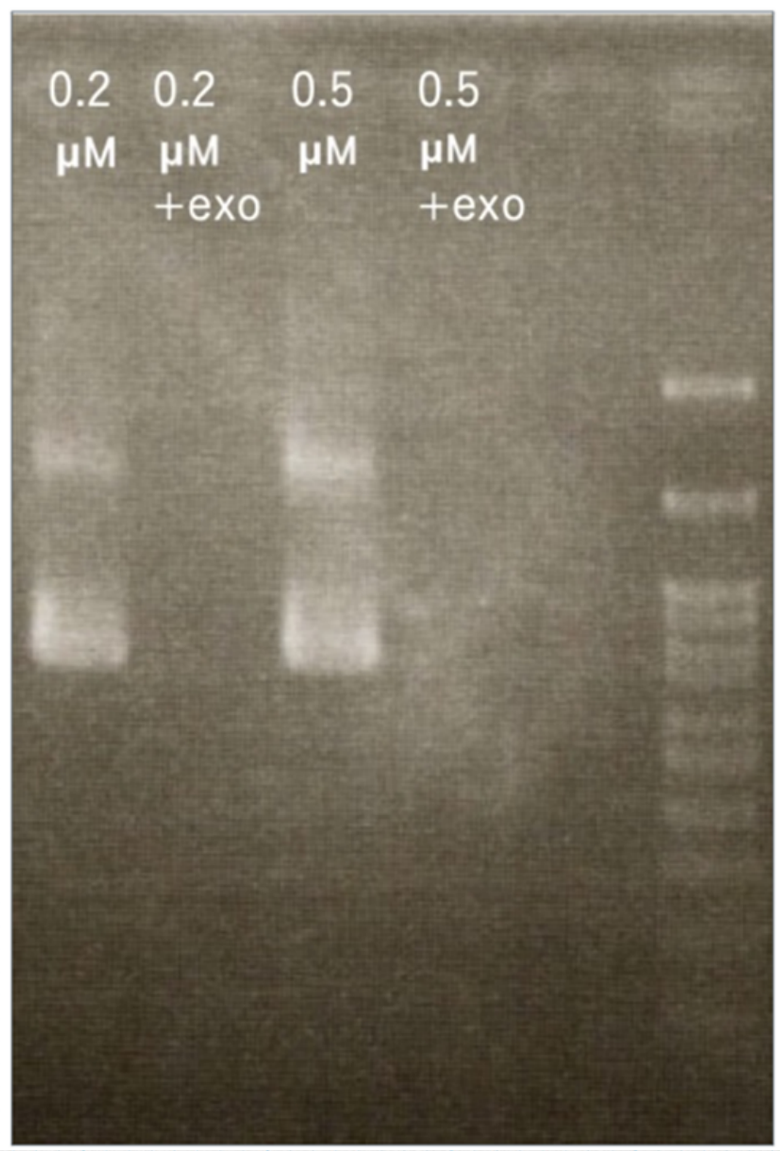
circular



linear

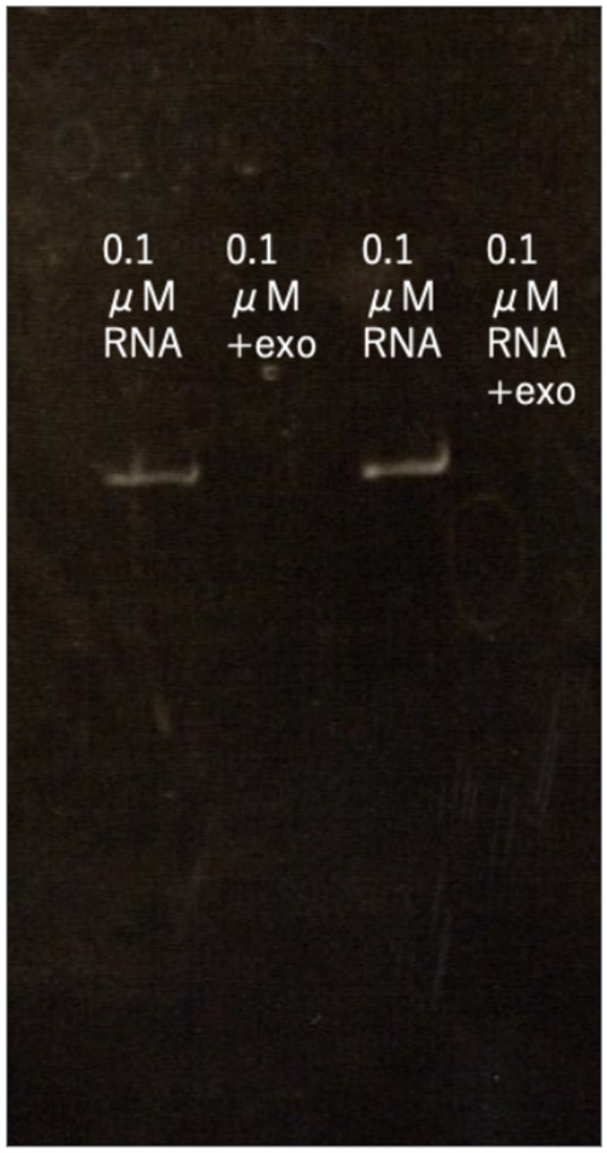
RNaseR, incubate 2 hr





Added RNase R. Line1-2 0.2 µM, Line3-4 0.5µM

Project



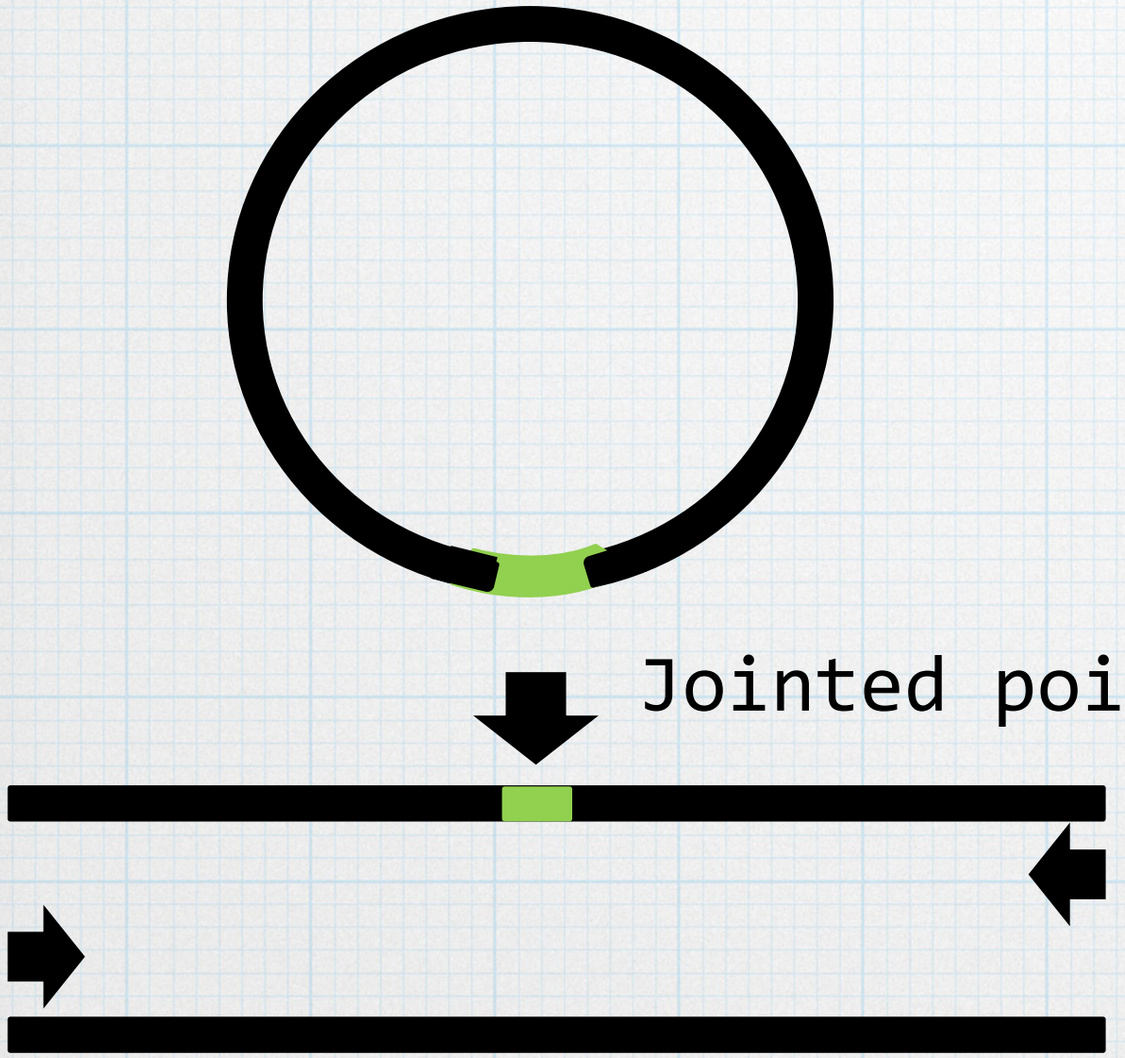
Short sammary

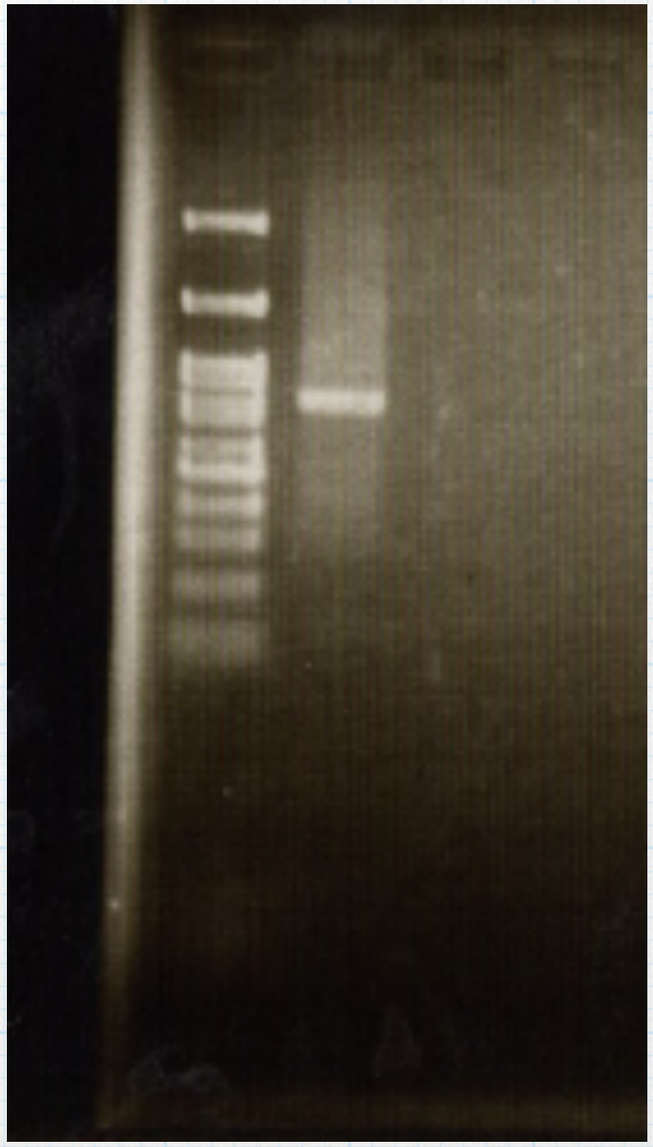
- Failed to confirm by circRNA agarose gel electrophoresis and denaturing PAGE.
- All staining used ethidium bromide.

Detection may be possible by using a fluorescent agent that easily stains RNA such as CYBER Green Ⅰ.

(Heike Summer1 et al.) Denaturing Urea Polyacrylamide Gel Electrophoresis(Urea PAGE)

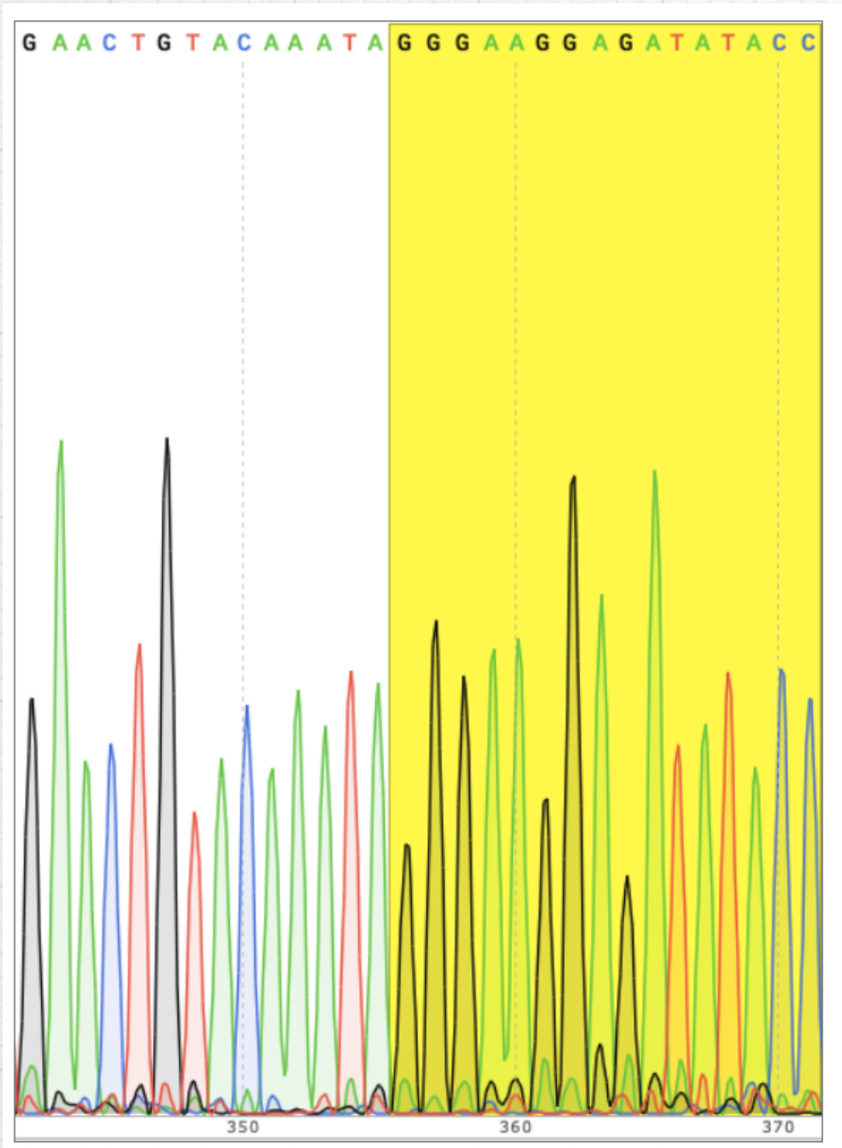
Project





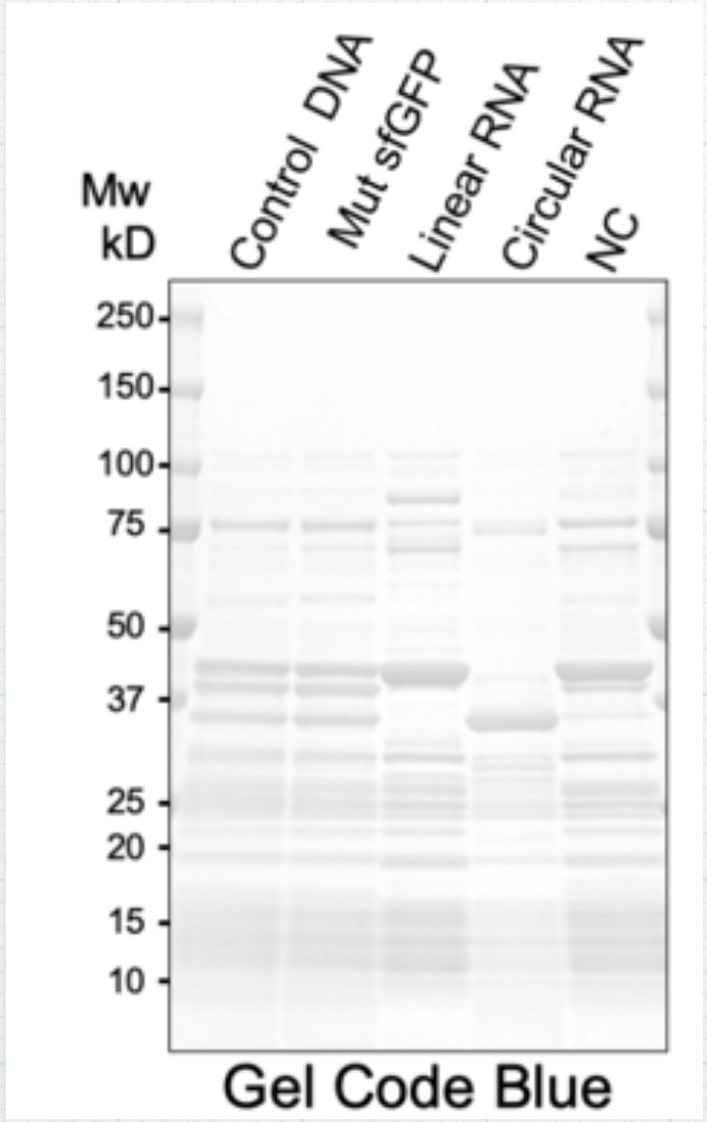
Line2 and 3 amplified RNA after reverse-transcription

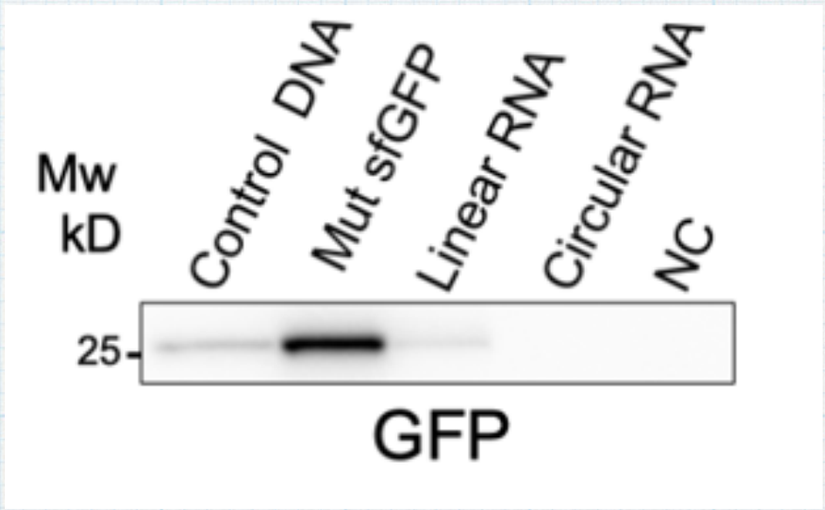
Project



Obtained unique sequence

Project


Mw
kD
Control DNA
Mut sfGFP
Linear RNA
Circular RNA
NC
250
150
100
75
50
37
25
20
15
10
Gel Code Blue

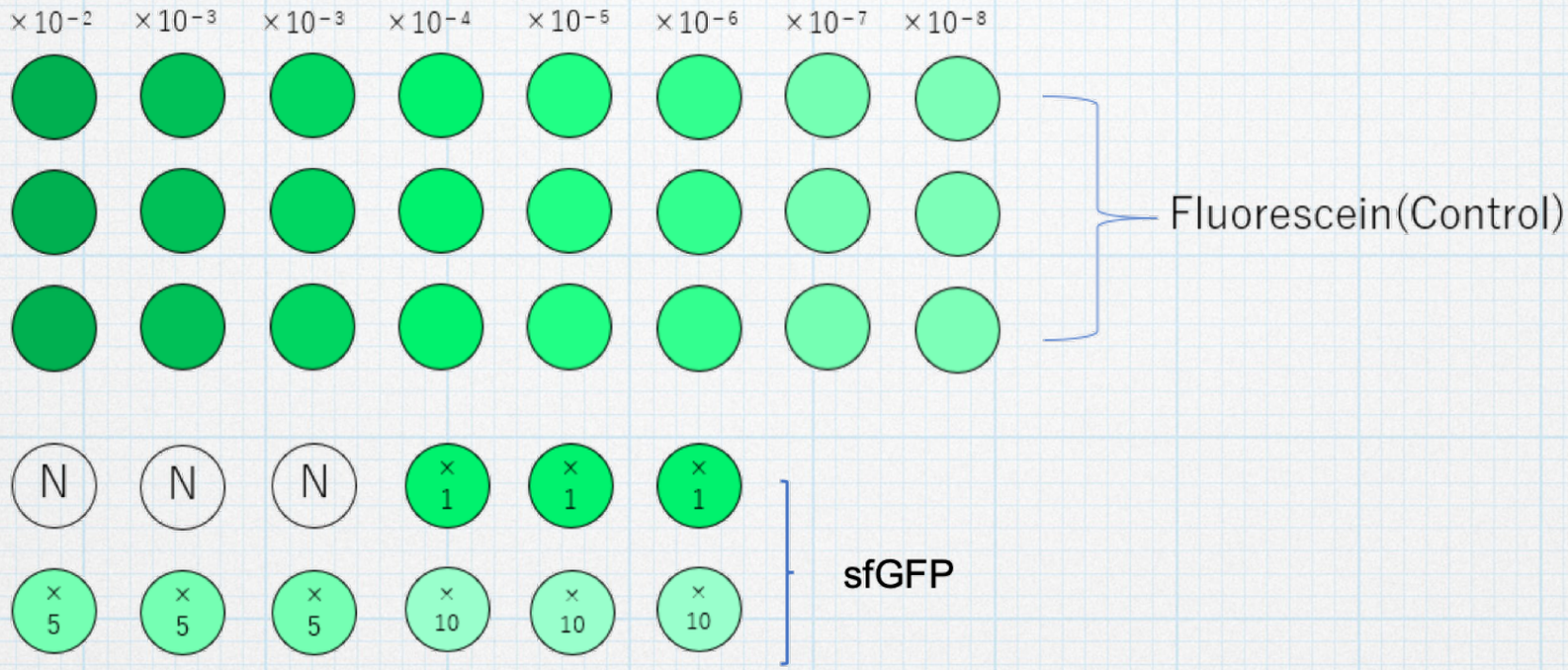

Mw
kD
Control DNA
Mut sfGFP
Linear RNA
Circular RNA
NC
25
GFP

Discussion and Future work

Discussion

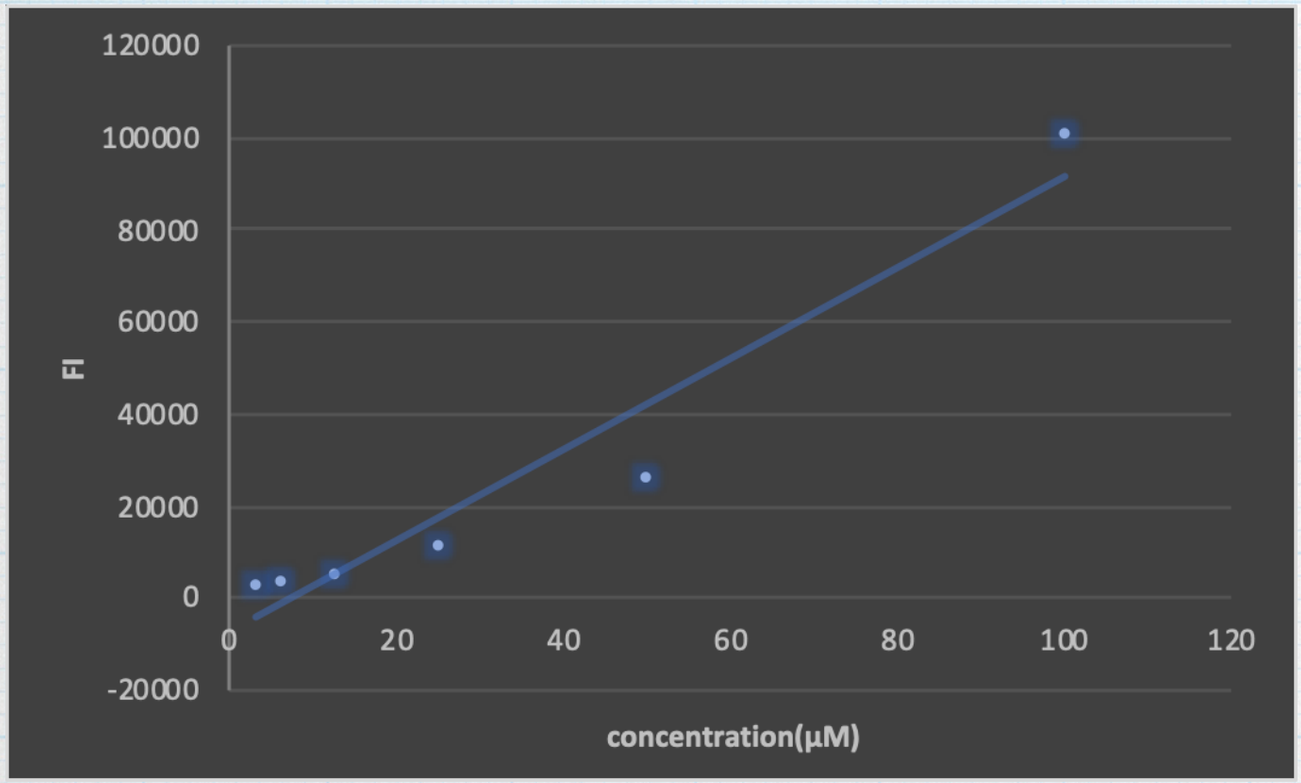
- From the result of the sequence, we can say that we sucessed in generated circular RNA.
- There was some troubles with RNA structure because proteins were not synthesized from circular RNA.
- Efficiency of circularization must be increased.
- Find new method to assay circular RNA.

Measurement of expression speed by Real-Time PCR

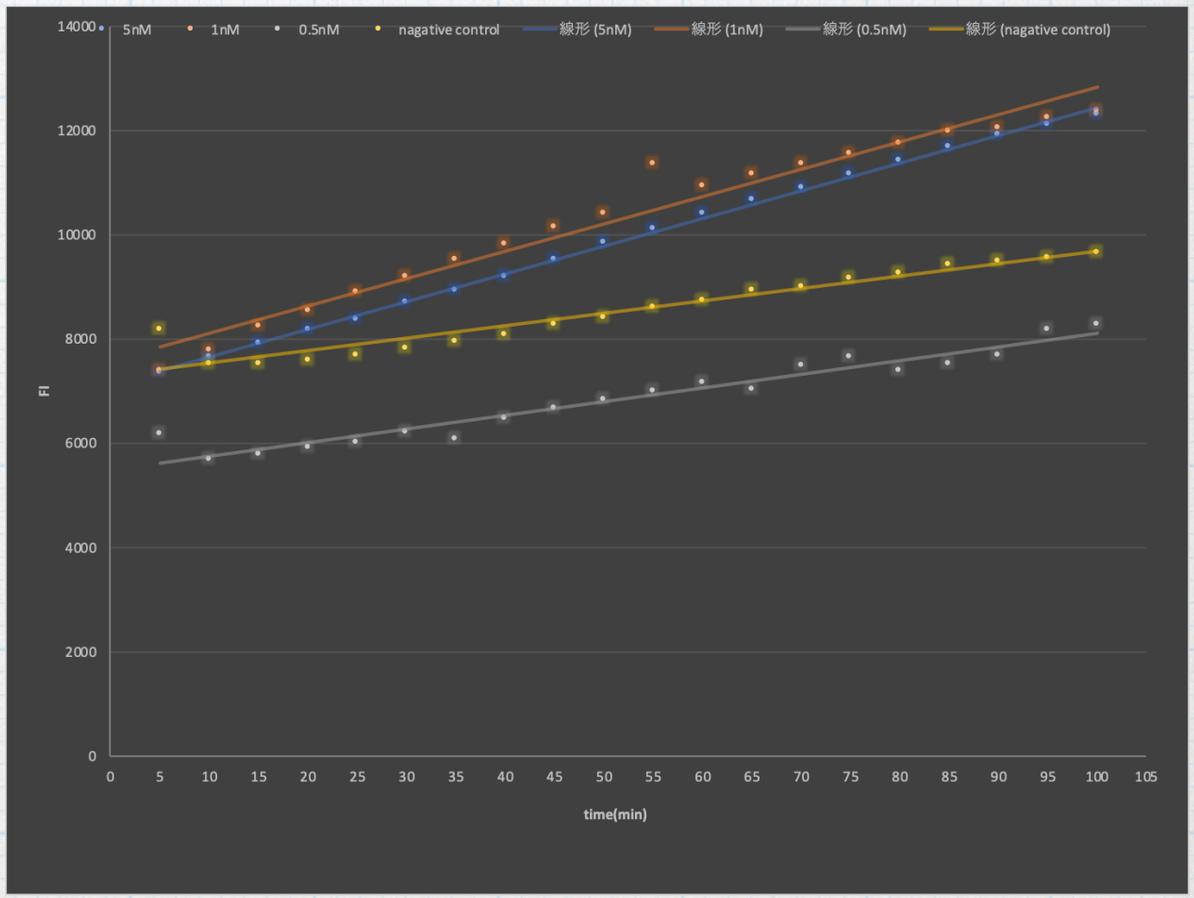


- we used myTXTL from Arbor Biosciences and provided plasmid as a positive control. (eGFP)
- FI(fluorescein intensity) of fluorescein at 8 different condition.
- Four conditions for the cell-free system, 5 nM of the plasmid, 1 nM, 0.5 nM and 0 nM (Water).

Measurement of expression speed by Real-Time PCR

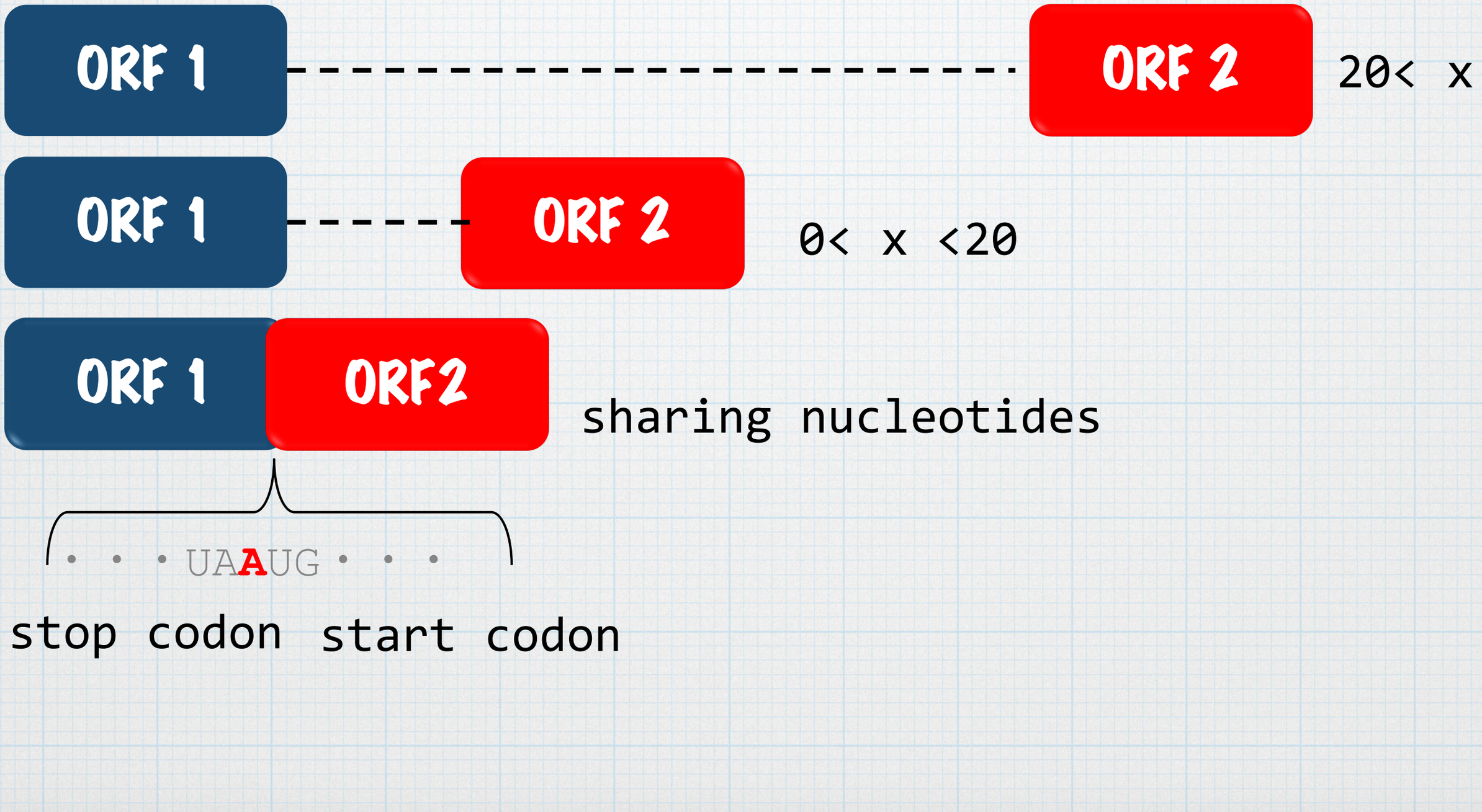


Calibration carve of fluorescein



Calibration carve of sfGFP

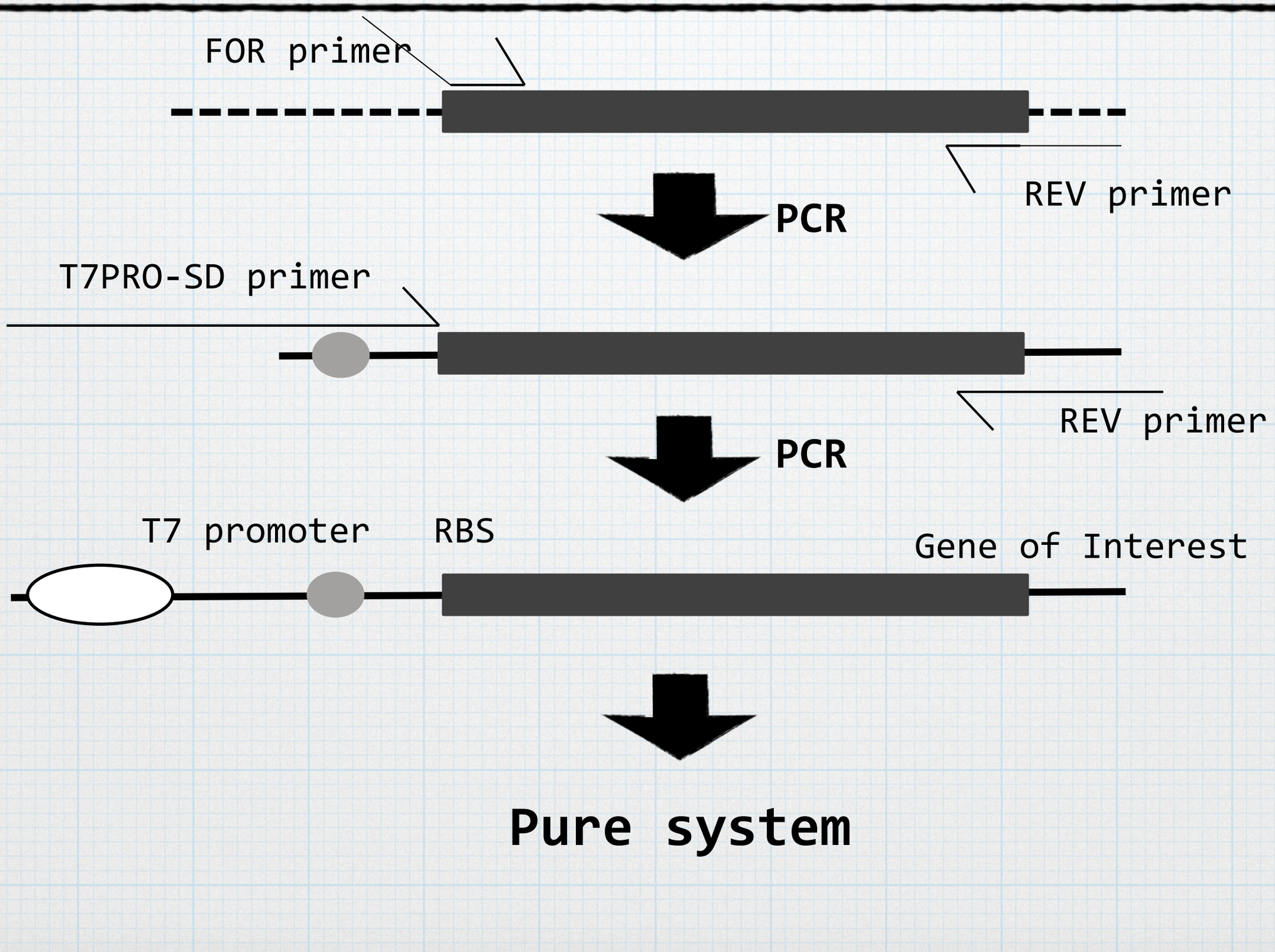
Future Work


ORF 1
ORF 2
20< x
ORF 1
ORF 2
0< x <20
ORF 1
ORF2
sharing nucleotides
• • • UAAUG • • •
stop codon
start codon

Characterization

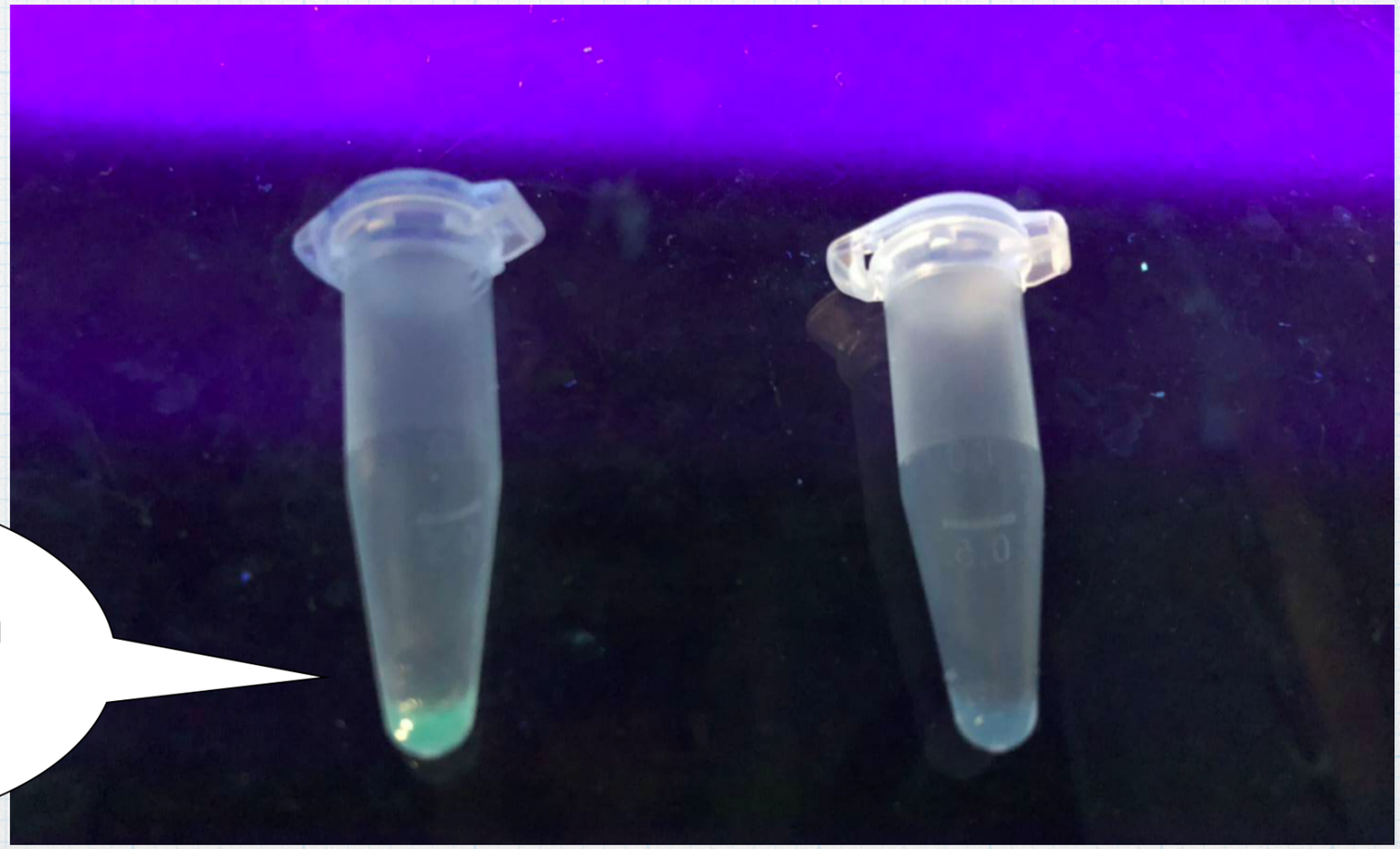
Characterised BBa_I746916 and checked Quantification of GFP and the expression of sfGFP by Western Blotting

Experiment PCR amplification



Pure system

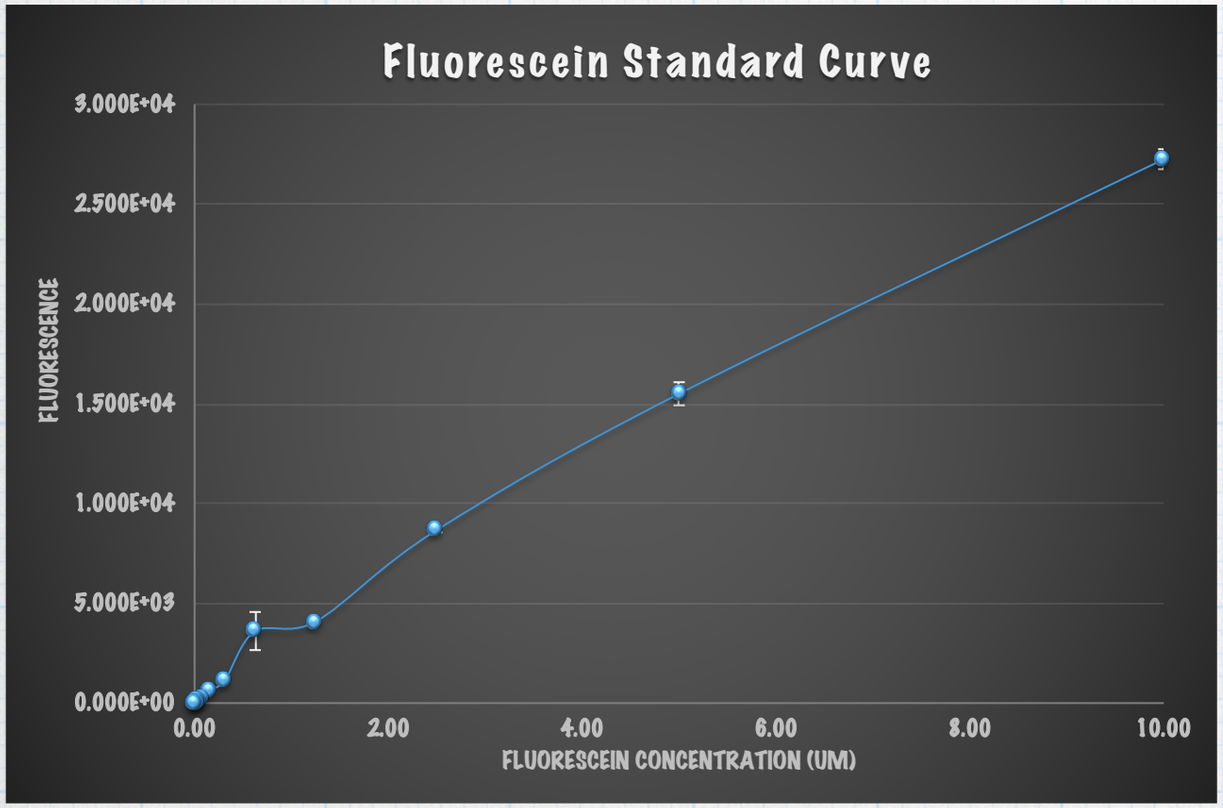
Result

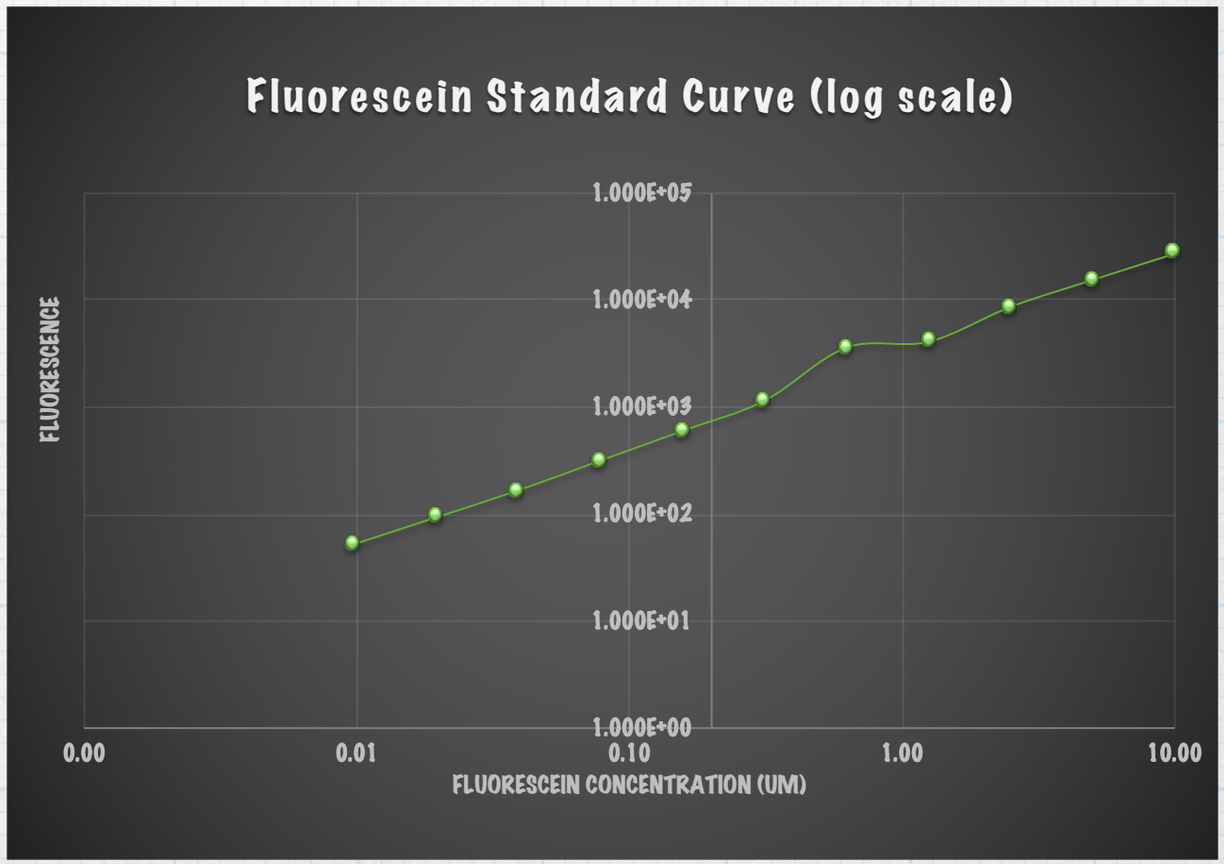


Left: BBa_I746916　　Right: Negative control (Water)

Result

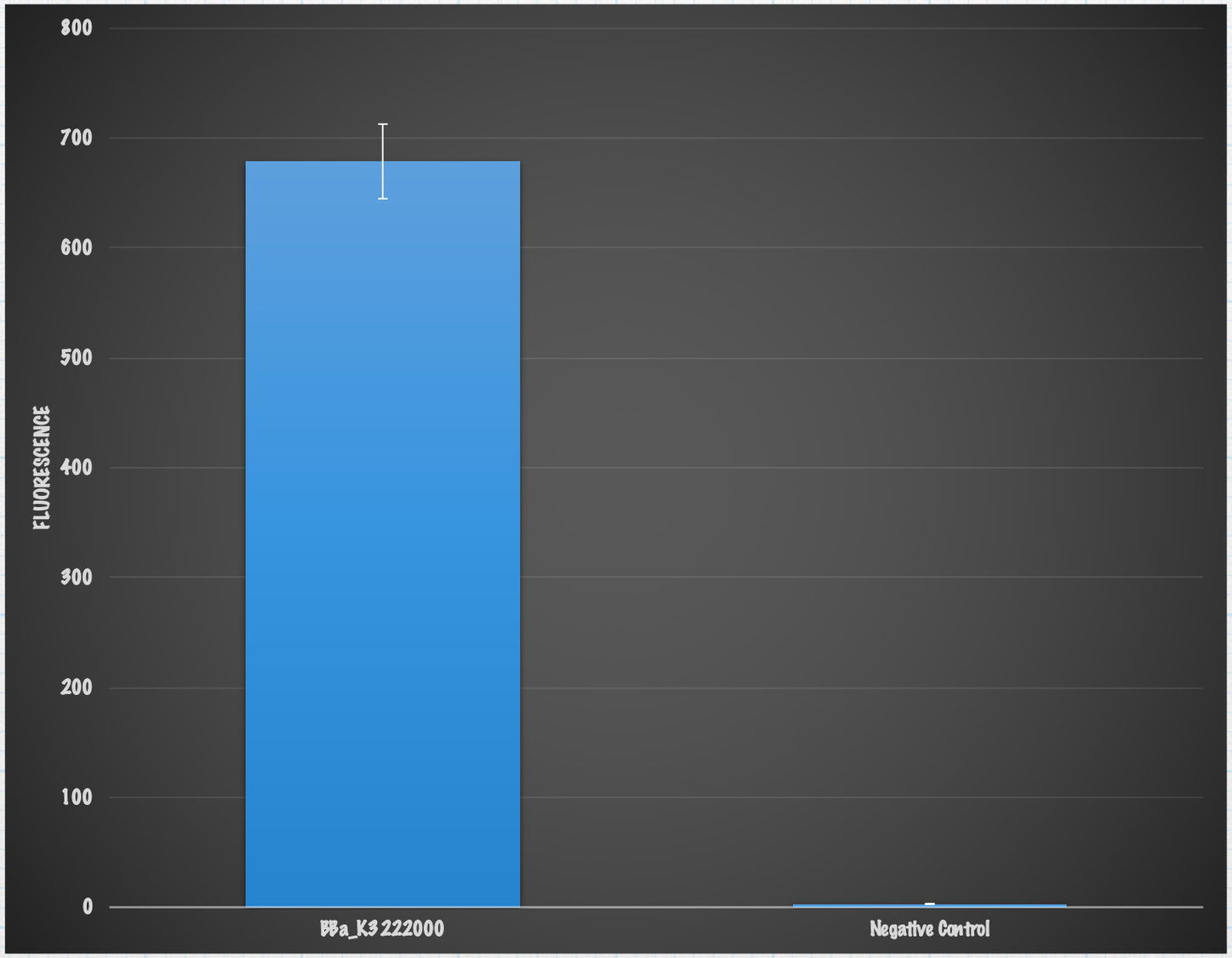
Fluorescein Calibration





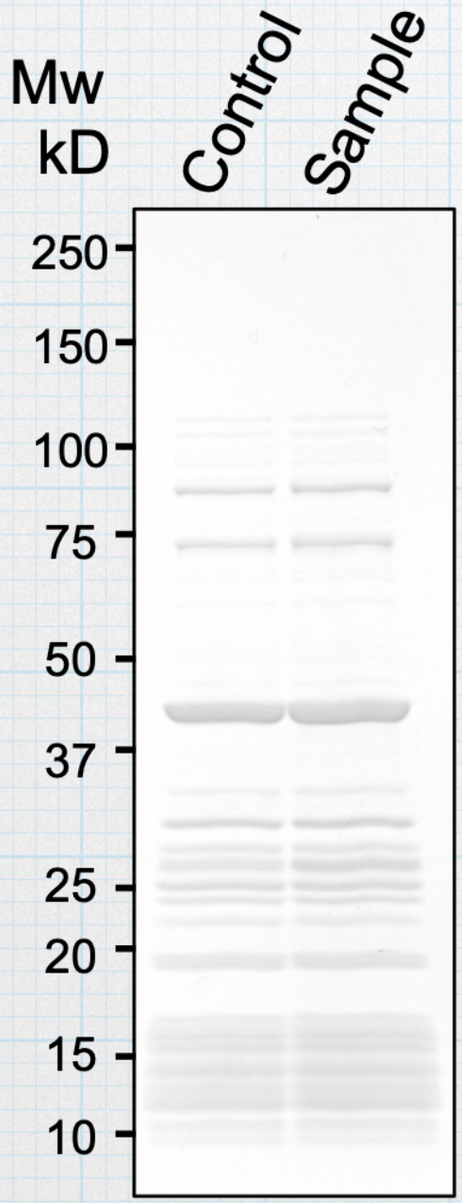
Result

Measuring the quantification of sfGFP with Microplate Reader

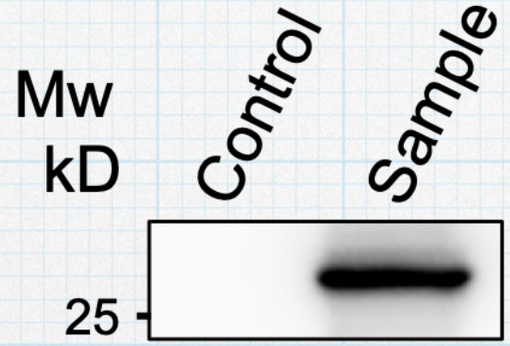


Result

Western Blotting



Gel Code Blue



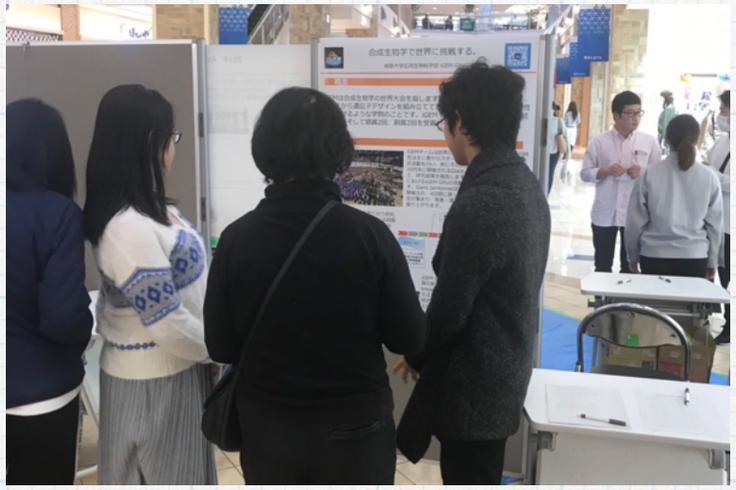
GFP

Confirmination of the band of sfGFP

Human Practice

Human practice

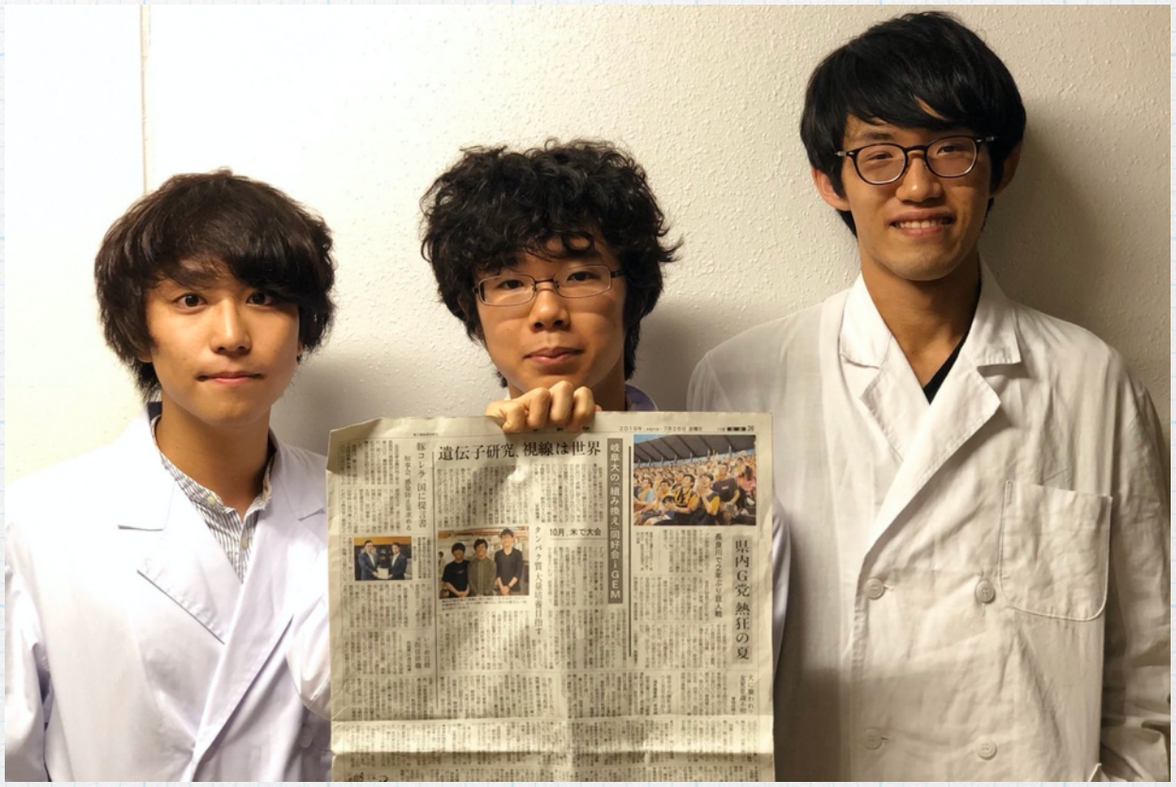
Education and Engagement



1. Exhibition of poster and flyers of iGEM Gifu TOKAI at Ibigawa Green Tea festival
2. Poster exhibition in Gifu University Fair
3. Poster session with high school students in Open Campus of Gifu University

Human practice

Education and Engagement



1.Getting financial support from Ito Scholarship
2.Our activities were introduced in Gifu Newspaper

Reference

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Acknowledgement

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Applied Microbiology laboratory

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Professor T.NAKAGAWA

Takashi KANAMORI, PhD

Rena MATSUMOTO, PhD

Professor H.ABE

Professor Y.KIZUKA

Support

President MORIWAKI

Gifu University

arbor biosciences

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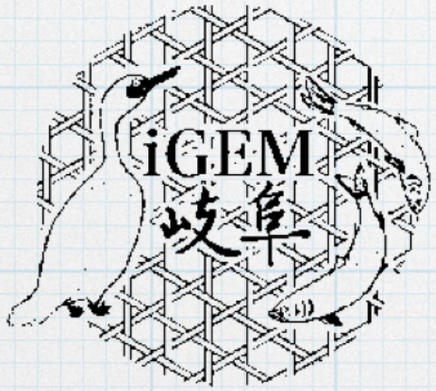
We appreciate all supports from the laboratory, the professors and companies.

Thank you for your understanding.



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