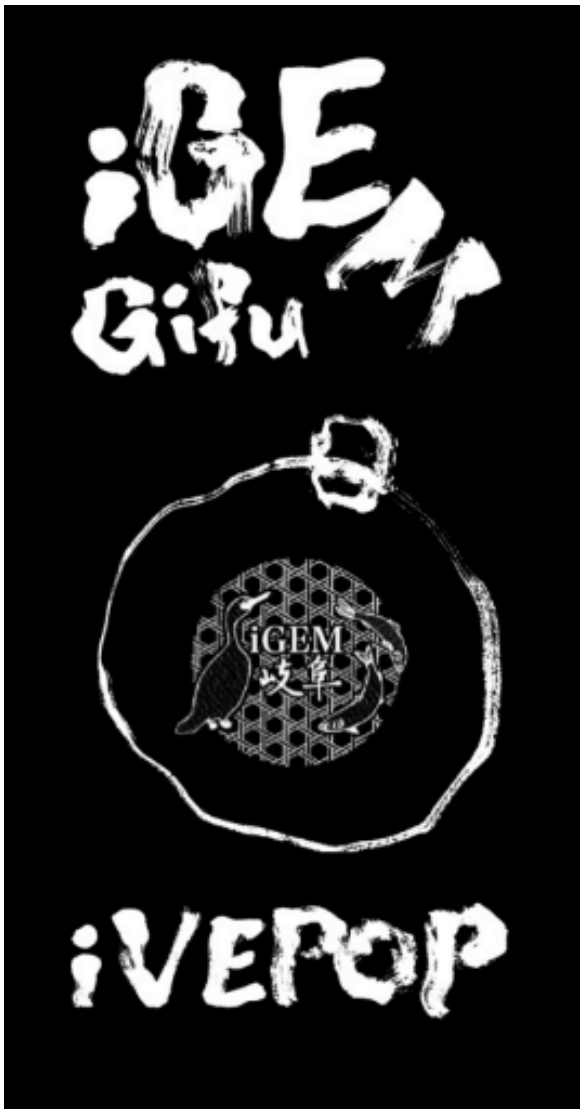

IGEM GIFU 2019

PROTOCOL

ABSTRACT

We, iGEM GIFU_TOKAI, focus on mRNA and changing its topological form into circular to create a new method for mass-production of protein in cell-free system this year. In the current research of circular RNA (circRNA) for protein production, expressing tandem-repeated protein was generated by circRNA without a stop codon. It shows circRNA has a potential ability that it can skip the rate-limiting process,binding ribosomes to mRNA of the central dogma of molecular biology. However, with conventional circRNA, functional protein cannot be translated because protein aggregation quickly occurs. Therefore, we decided to use translation-coupling system, which is found in operons of bacteria to produce monomer protein from circRNA. With applying it to circRNA, ribosomes repeat translation-coupling phenomenon in circRNA and are expected to express monomer protein. Our final goal is to produce functional proteins such as antibodies more efficiently and cheaper in cell-free system to provide medicaments consistently.

Edited by Ryo NIWA, Yuichiro IKAGAWA, Fumiya ITO
in 2019

iGEM GIFU TOKAI 2019 PROTOCOL
iVEPOP
I. PCR - DNA amplification -
Material
Template DNA
GIFU_TOKAI_1

```
Taatacgactcactatagggaaggagatataccaatgcgt
aaaggcgaagagctgttcactggtgtcgtccctattctgg
tggaactggatggtgatgtcaacggtcataagttttccgt
gcgtggcgagggtgaaggtgacgcaactaatggtaaactg
acgctgaagttcatctgtactactggtaaactgccggtac
cttggccgactctggtaacgacgctgacttatggtgttca
gtgctttgctcgttatccggaccatatgaagcagcatgac
ttcttcaagtccgccatgccggaaggctatgtgcaggaac
gcacgatttcctttaaggatgacggcacgtacaaaacgcg
tgcggaagtgaaatttgaaggcgataccctggtaaaccgc
attgagctgaaaggcattgactttaaagaagacggcaata
tcctgggccataagctggaatacaattttaacagccacaa
tgtttacatcaccgccgataaacaaaaaaatggcattaaa
gcgaattttaaaattcgccacaacgtggaggatggcagcg
tgcagctggctgatcactaccagcaaaacactccaatcgg
tgatggtcctgttctgctgccagacaatcactatctgagc
acgcaaagcgttctgtctaaagatccgaacgagaaacgcg
atcatatggttctgctggagttcgtaaccgcagcgggcat
cacgcatggtatggatgaactgtacaaata
```

Primers
Cont_Fw

```
aaggagatataccaatgcgtaaaggcgaag
```

Cont_Rv

```
ggattagttattcattattatttgtacagttcat
```

T7_SD_Pro Fw

```
gaaattaatacgactcactatagggagaccacaacggttt
ccctctagaaataattttgtttactttaagaaggagatat
acca
```

Cont_No1_New Fw

```
aggagatataccaatgcgtaaaggcgaggag
```

Cont_No3_Fw

```
gaaattaatacgactcactatagggaaacc
```

T7_Abe_Fw

```
Cgcggatcctaatacgactcactatag
```

Rev_2-o-methyl_primer

```
atttgtacagttcatccat
```

Polymerase
KAPA HiFi (KAPA BIOSYTEMS)

Protocol
Control DNA
All Primers and template DNA should be tapped and centrifuged briefly before using to prevent loss and/or decrement of PCR product that amplified using the reagents.

PCR mixture

Template DNA	1uL
Polymerase (KAPA HiFi)	12.5uL
Primer (Cont_Fw)	1.5uL
Primer (Cont_Rv)	1.5uL
Water	8.5uL
Total vol.	25uL

Run the PCR

95℃	3 min
98℃	20 sec
60℃	15 sec
72℃	1 min 30 sec
72℃	3 min
4℃	∞
30 cycles	

Table.1 control PCR mixture and PCR cycling protocol I

The amplified control DNA fragments must be amplified again to remove the recognition site for *Sap I*, type IIS restriction enzyme by Cont_No1_New Fw

(change the Fw primer) after amplified template using Cont_Fw primer and Cont_Rv primer.

PCR mixture	
Template DNA	1uL
Polymerase (KAPA HiFi)	12.5uL
PrimerFw (Cont_No1_New Fw)	1.5uL
PrimerRv(Cont_Rv)	1.5uL
Water	8.5uL
Total vol.	25uL

Run the PCR	
95℃	3 min
98℃	20 sec
60℃	15 sec
72℃	1 min 30 sec
72℃	3 min
4℃	∞
30 cycles	

Moreover, the PCR product has to amplify by T7PRO_SD(change the Fw primer) after amplified template using Cont_Fw primer and Cont_Rv primer. When running the PCR, we have to change the annealing temperature from 58℃ to 60℃ (touch-down PCR) because T7_SD_PRO primer has longer adapter region than overlapping region, it is difficult to hybridize to template DNA with usual annealing temperature.

PCR mixture	
Template DNA	2 uL
Polymerase (KAPA HiFi)	25 uL
PrimerFw (T7PRO_SD)	3 uL
PrimerRv(Cont_Rv)	3 uL
Water	17 uL
Total vol.	50 uL

Run the PCR	
95℃	3 min
98℃	20 sec
58→60℃	15 sec
72℃	1 min 30 sec
72℃	3 min
4℃	∞
30 cycles	

Finally, the product must be amplified with Cont_No3_Fw primer to eliminate *BsaI*, recognition site as type IIS restriction enzyme.

PCR mixture	
Template DNA	2 uL
Polymerase (KAPA HiFi)	25 uL
PrimerFw (Cont_No3_Fw)	3 uL
PrimerRv(Rev_2-o-methyl_Primer)	3 uL
Water	17uL
Total vol.	50uL

Run the PCR	
95℃	3 min
98℃	20 sec
60℃	15 sec
72℃	1 min 30 sec
72℃	3 min
4℃	∞
30 cycles	

Table.2 control PCR mixture and PCR cycling protocol II

Medium SD

PCR mixture	
Template DNA	2 uL
Polymerase (KAPA HiFi)	25 uL
PrimerFw (T7_Abe_Fw)	3 uL
PrimerRv(Rev_2-o-methyl_Primer)	3 uL
Water	17uL
Total vol.	50uL

Run the PCR	
95℃	3 min
98℃	20 sec
58℃	15 sec
72℃	1 min 30 sec
72℃	3 min
4℃	∞
30 cycles	

Table.3 medium-SD PCR mixture and PCR cycling protocol

II. Transcription for DNA

We transcript using MEGAscript® by Thermo Fisher Bioscience®. Follow protocol for MEGAscript® when we transcript control DNA fragment (it will be used still linear fragment after transcription). However, guanosine monophosphate is added to MEGAscript® mix when Full-SD DNA fragment and Weak-SD DNA fragment are transcribed because it needs the procedure to circularize using T4 RNA ligase 2. The reason will be described next part.

Material

Enzyme Mix
10X Reaction Buffer
ATP Solution
CTP Solution
GTP Solution
UTP Solution
Guanosine monophosphate (GMP)
Nuclease-free Water
Template DNA

Amount	Component
to 20 uL	Nuclease-free Water
2 uL	ATP
2 uL	CTP
2 uL	GTP
2 uL	UTP
2 uL	10× Reaction Buffer
(1 uL)	(optional)[α-^{32}P]UTP as a trancer
0.1-1 ug	linear template DNA
2 uL	Enzyme Mix

Table.6 protocol for transcription with control DNA

Use 0.1–0.2μg PCR-product template or ~1μg linearized plasmid template.

Important

All reagents should be microfuged briefly before opening to prevent loss and/or contamination of material that may be present around the rim of the tube.

Control DNA

Follow original protocol for MEGAscript®.

Amount	Component
to 20 uL	Nuclease-free Water
2 uL	ATP
2 uL	CTP
2 uL	GTP
2 uL	UTP
2 uL	10× Reaction Buffer
(1 uL)	(optional)[α-^{32}P]UTP as a trancer
0.1-1 ug	linear template DNA
2 uL	Enzyme Mix

Table.8 Transcription mixture for control DNA

1. Thaw the frozen reagents Place the RNA Polymerase Enzyme Mix on ice.
2.Vortex the 10X Reaction Buffer and the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP) until they are completely in solution.

3. If they thawed, store the ribonucleotides on ice, but keep the 10X Reaction Buffer at room temperature while assembling the reaction.

4. The spermidine in the 10XReaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.

5. Add the 10XReaction Buffer after the water and the ribonucleotides are already in the tube. The following amounts are for a single 20µL reaction. Reactions may be scaled up or down if desired.

6. Gently flick the tube or pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.

7. Incubate at 37° C, 2–4hr The first time a new template is transcribed, the recommended incubation time is 2–4hours.

Transcription for circular RNA (medium-SD)

Add guanosine monophosphate to mixture when transcripts medium-SD DNA, weak-SD DNA, and strong-SD DNA, and follow the present protocol otherwise.

Amount	Component
to 20 uL	Nuclease-free Water
2 uL	ATP
2 uL	CTP
2 uL	GTP
2 uL	UTP
2 uL	10× Reaction Buffer
(1 uL)	(optional)[α-^{32}P]UTP as a trancer
0.1-1 ug	linear tem late DNA
2 uL	Enzym e M ix
75 m M	GM P

Table.8 Transcription mixture for medium-SD

1. Thaw the frozen reagents Place the RNA Polymerase Enzyme Mix on ice.

2. Vortex the 10X Reaction Buffer and the 4 ribonucleotide solutions (ATP, CTP, GTP, and UTP) until they are completely in solution.

3. If they thawed, store the ribonucleotides on ice, but keep the 10XReaction Buffer at room temperature while assembling the reaction.

4. The spermidine in the 10XReaction Buffer can coprecipitate the template DNA if the reaction is assembled on ice.

5. Add the 10X Reaction Buffer after the water and the ribonucleotides are already in the tube. The following amounts are for a single 20µL reaction. Reactions may be scaled up or down if desired.

6. Gently flick the tube or pipette the mixture up and down gently, and then microfuge tube briefly to collect the reaction mixture at the bottom of the tube.

7. 2–4hr the first time a new template is transcribed, the recommended incubation time is 2–4 hours.

III. Eliminate the template DNA

Material

TURBO DNase

Protocol

1. Add 1µL TURBO DNase, and mix well (the reaction may be viscous).

2. Incubate at 37° C for 15min.

IV. Purification RNA

Reagents

Binding Solution

Wash Solution Concentrate (Add 20ml 100% ethanol before use)

Ammonium Acetate

Gear

Elution Solution

Filter Cartridges

Elution Tubes

RNA should be purified by MEGAclear®. Follow the

protocol.

Protocol

＊Prepare the Wash Solution

Add 20 mL of ACS grade 100% ethanol to the bottle labeled Wash Solution Concentrate. Mix well. Place a check in the box on the label to indicate that the ethanol was added. With the ethanol, this solution will be referred to as Wash Solution.

1. Bring the RNA sample to 100 µL with Elution Solution. Mix gently but thoroughly.

2. Add 350 µL of Binding Solution Concentrate to the sample. Mix gently by pipetting.

3. Add 250 µL of 100% ethanol to the sample. Mix gently by pipetting.

4. Apply the sample to the filter:
Centrifuge users:
a. Insert a Filter Cartridge into 1 of the Collection and Elution Tubes supplied.
b. Pipet the RNA mixture onto the Filter Cartridge.
c. Centrifuge for ~15 sec to 1 min, or until the mixture has passed through the filter. Centrifuge at RCF 10,000–15,000 × g (typically 10,000–14,000 rpm). Spinning harder than this may damage the filters.
d. Discard the flow-through and reuse the Collection and Elution Tube for the washing steps.

5. Wash with 2 × 500 µL Wash Solution.
a. Apply 500 µL Wash Solution. Draw the Wash Solution through the filter as in the previous step.
b. Repeat with a second 500 µL aliquot of Wash Solution.
c. After discarding the Wash Solution, continue centrifugation or leave the Filter Cartridge on the vacuum manifold for 10–30 sec to remove the last traces of Wash Solution.

6. Elute RNA from the filter with 50 µL Elution Solution using one of the methods described below; they are equivalent in terms of RNA recovery.
• RNA elution option 1
a. Place the Filter Cartridge into a new Collection/Elution Tube.
b. Apply 50 µL of Elution Solution to the center of the Filter Cartridge. Close the cap of the tube and incubate in a heat block set to 65–70° C for 5–10 min.
c. Recover eluted RNA by centrifuging for 1 min at RT (RCF 10,000–15,000 × g).
d. To maximize RNA recovery, repeat this elution procedure with a second 50 µL aliquot of Elution Solution. Collect the eluate into the same tube.
c. To maximize RNA recovery, repeat this elution procedure with a second preheated 50 µL aliquot of Elution Solution. Collect the eluate into the same Collection/Elution Tube.
Note: If glass fibers are observed in your sample, they can be removed by centrifuging the sample briefly and then transferring the RNA to a new tube.

V. Circularization for RNA

Material

RNase inhibitor
T4 RNA ligase
×10 reaction buffer
Guide DNA

Circularize the RNA using T4 RNA ligase 2 with guide DNA, so the fragment is circularized to be efficiently by closing each termination for the RNA. Add RNase inhibitor to avoid digestion for the circular RNA.

Amount	Component
Up to 40 uL	Nuclease-Free Water
3 uL	template RNA
2 uL (4 unit)	RNase Inhibitor
1 uL	T4 RNA Ligase 2
4 uL	×10 reaction Buffer
2 uL	Guide DNA

Table.9 Circularization for RNA

1. Thaw the reagents and the linear RNA template on ice, and place on -20℃.
2. Vortex or tapping the reagents and centrifuge.
3. Add Nuclease-Free water, Guide DNA, and template RNA to clean PCR tube, and it heat 90℃, 5 minutes to anneal both strands.
4. Mix reagents other than T4 RNA ligase2, ×10 reaction buffer in the tube.
5. Incubate 37℃, 3 h after heat 60℃, 5 minutes to anneal guide DNA and template.

VI. Purification for circular RNA

IV. Purification RNA

Material

Reagents

Binding Solution
Wash Solution Concentrate (Add 20ml 100% ethanol before use)
Ammonium Acetate

Gear

Elution Solution
Filter Cartridges
Elution Tubes

RNA should be purified by MEGAclear®. Follow the protocol.

Protocol

＊Prepare the Wash Solution
Add 20 mL of ACS grade 100% ethanol to the bottle labeled Wash Solution Concentrate. Mix well. Place a check in the box on the label to indicate that the ethanol was added. With the ethanol, this solution will be referred to as Wash Solution.

1. Bring the RNA sample to 100 µL with Elution Solution. Mix gently but thoroughly.

2. Add 350 µL of Binding Solution Concentrate to the sample. Mix gently by pipetting.

3. Add 250 µL of 100% ethanol to the sample. Mix gently by pipetting.

4. Apply the sample to the filter:
Centrifuge users:
a. Insert a Filter Cartridge into 1 of the Collection and Elution Tubes supplied.
b. Pipet the RNA mixture onto the Filter Cartridge.
c. Centrifuge for ~15 sec to 1 min, or until the mixture has passed through the filter. Centrifuge at RCF 10,000–15,000 × g (typically 10,000–14,000 rpm). Spinning harder than this may damage the filters.
d. Discard the flow-through and reuse the Collection and Elution Tube for the washing steps.

5. Wash with 2 × 500 µL Wash Solution.
a. Apply 500 µL Wash Solution. Draw the Wash Solution through the filter as in the previous step.
b. Repeat with a second 500 µL aliquot of Wash Solution.
c. After discarding the Wash Solution, continue centrifugation or leave the Filter Cartridge on the vacuum manifold for 10–30 sec to remove the last traces of Wash Solution.

6. Elute RNA from the filter with 50 µL Elution Solution using one of the methods described below; they are equivalent in terms of RNA recovery.
• RNA elution option 1

a. Place the Filter Cartridge into a new Collection/Elution Tube.
b. Apply 50 µL of Elution Solution to the center of the Filter Cartridge. Close the cap of the tube and incubate in a heat block set to 65–70° C for 5–10 min.
c. Recover eluted RNA by centrifuging for 1 min at RT (RCF 10,000–15,000 × g).
d. To maximize RNA recovery, repeat this elution procedure with a second 50 µL aliquot of Elution Solution. Collect the eluate into the same tube.
c. To maximize RNA recovery, repeat this elution procedure with a second preheated 50 µL aliquot of Elution Solution. Collect the eluate into the same Collection/Elution Tube.
Note: If glass fibers are observed in your sample, they can be removed by centrifuging the sample briefly and then transferring the RNA to a new tube.

VII. Eliminate linear RNA by RNase R

Material

RNase R
×10 reaction Buffer
RNA mixture (include linear RNA and circular RNA)

Amount	Component
Up to 20 uL	Nuclease-Free Water
1-10 ug	RNA mixture
2 uL	RNase R
2 uL	×10 RNase R reaction buffer

1. Mix reagents and RNA mixture at nuclease-free tube.
2. Incubate 37℃, 15 min.

VIII. Expression for Protein in vitro

Reagents

myTXTL LS70 Master Mix (Arber Bioscience)
PURE frex
Solution I
Solution II
Solution III

We expressed superfolder GFP via *in vitro* translation system such as myTXTL® and PURE frex®. myTXTL® is crude cell extract cell-free system that leach transcription and translation factor from *E. coli* . On the other hand, PURE frex® was sorted reconstitution cell-free system that these are using purified translation factor such as initiation, elongation, and termination. We attempted to compare both cell-free system.

myTXTL®

We add template RNA followed myTXTL.

Amount	Component
9 uL	LS70 Master Mix
3 uL	Template RNA

2. Completely thaw the myTXTL LS70 Master Mix on ice. Keep reagents on ice till use.
3. Directly before use, vortex the myTXTL LS70 Master Mix for 2-3 seconds and briefly spin down.
4. Add template RNA and Master Mix to nuclease free tube, then template RNA must be added carefully to a final concentration of 20 nM.
5. Gently vortex the reaction mixture for 2-3 seconds and briefly centrifuge the assembled myTXTL reaction to collect the entire volume at the bottom of the tube.
6. Incubate the myTXTL reaction(s) for up to 16 h at 29°C.

PURE frex®

Followed protocol for PUREfrex

Amount	Component
7-X uL	Nuclease-Free water
10 uL	Solution I
1 uL	Solution II
2 uL	Solution III
X uL	template (1 kbp : 0.5~3ng/uL)

1. Completely thaw the Solution I in room temperature

or 37℃ at 1 minute, and store on ice.

2. Solution II and Soluition III should thaw on ice.

3. Vortex the reagents for few seconds and briefly spin down.

4. Mix all reagents and template RNA/DNA in nuclease free tube.

5. Incubate 37℃, 2~6h.

IX. Assay

We used bioanalyzer (Agilent Technology) and real-time PCR (Thermo Fischer Scientific) as assay, also SDS-PAGE and western blotting was performed to identify the protein.