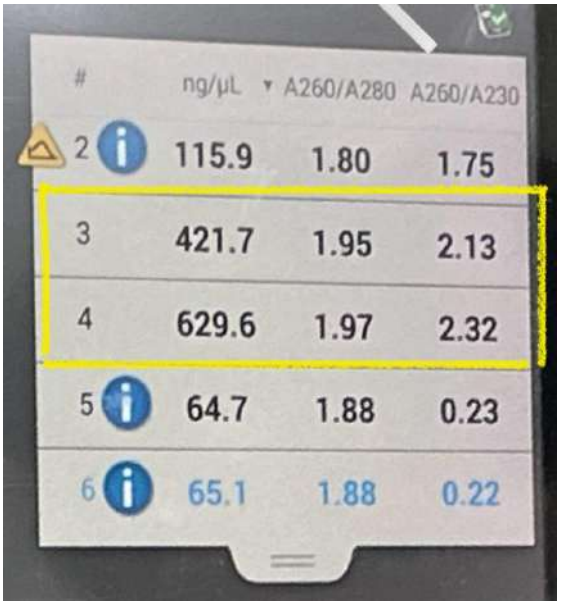
P. canaliculata HSP70 Gene Cloning Experiment

Day 1 (2024/07/01)

RNA extraction

1. Place RNA tissue in an homogenizing tube containing beads that facilitate the Trizol to homogenize the tissue. Homogenized tissue is observed to have a milky pink foam-like texture
2. Added chloroform and centrifuged the sample, creating three distinct layers: a top transparent layer containing RNA (aqueous phase) , a white thin and opaque middle layer (interface), and a bottom layer with protein and DNA with a clear pink color (organic phase).
3. Transferred the aqueous phase containing the RNA into an Eppendorf tube.
4. Added isopropanol which precipitates RNA. After centrifugation, an opaque white precipitation called a pellet appeared at the bottom of the tube
5. added 75% ethanol for cleaningthe pellet
6. Dried the sample until it became transparent.
7. Used DEPC treated water to suspend the sample
8. Used a nanodrop to measure the concentration as well as the quality of the collected RNA sample

Data
concentration: 525 ng/μL
A260/280: 1.96
A260/230: 2.23



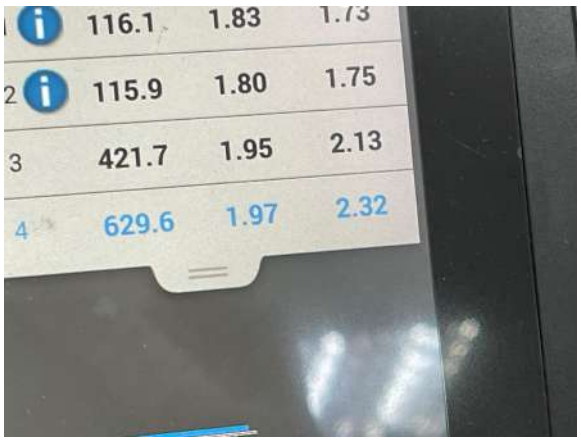
our results indicate that the RNA sample is of good quality

Agarose Gel electrophoresis

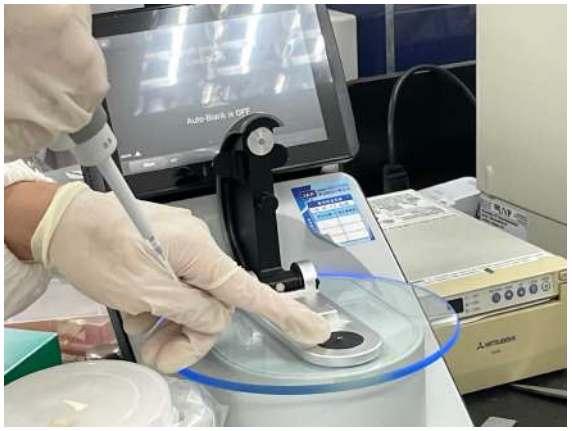
1. Combined TAE buffer and agarose powder (0.25g of agarose in 25mL TAE) and microwaved the solution until boiling with bubbles
2. incubated in 60°C water bath and poured into a gel tray with a well comb to solidify and form the gel
3. transferred the gel into a container and added 100mL of TAE buffer and 10µL of SYBR gold, which visualizes DNA under UV light, and placed it on a shaker at 50 RPM for 30 minutes
4. added TAE buffer to the electrophoresis apparatus and completely submerge the gel that was placed in the apparatus
5. Took out 2µL of the RNA sample, added RNA loading dye, mixed well, spin down and then loaded it into the wells
9. ran the electrophoresis under 100V. Observation: the dye moved away from the wells which could be identified by the presence of the loading dye
10. observed the movement of RNA on a UV cabinet. Observation: three distinct bands were visible.

Reverse transcription

1. Combined 5µL of the RNA sample with 1µL of random primer, 1µL of dNTPs, and 5µL of DEPC treated water
    a. random primer to initiate the reaction
    b. dNTPs (deoxynucleotide triphosphates) to provide the corresponding nucleotide for the template strand
    c. DEPC (diethylpyrocarbonate) treated water to exclude any RNase enzymes that could be present in regular water (RNase enzymes can cause degradation of the RNA)
2. Placed the mixture in the PCR machine from 65°C for 5 minutes and then cooled down to 4°C to denature the RNA and allow the random primer to more easily anneal to the RNA template.
3. Added 4µL first-strand buffer**,** 2µL 0.1M DTT, 1µL RNaseOUT, 1µL RNA transcriptase to the mixture
    a. first strand buffer to provide a more favorable environment for the reverse transcriptase enzyme to function more efficiently
    b. DTT (dithiothreitol) to prevent the DNA from forming dimer
    c. RNaseOUT (RNase inhibitor) to prevent the degradation of the RNA
    d. Reverse transcriptase enzymes to synthesize complementary nucleotides from the RNA template. (Convert RNA into cDNA)
4. mixed the mixture thoroughly using a pipette
5. Incubate the sample in the PCR machine under 25°C for 10 minutes then 42°C for an hour and then 70°C for 5 minutes and cool down at 4°C
6. final cDNA sample was stored at -20°C


116.1 1.83
115.9 1.80 1.75
421.7 1.95 2.13
629.6 1.97 2.32


Sample 5
Load #5
Blank
Measure
End Experiment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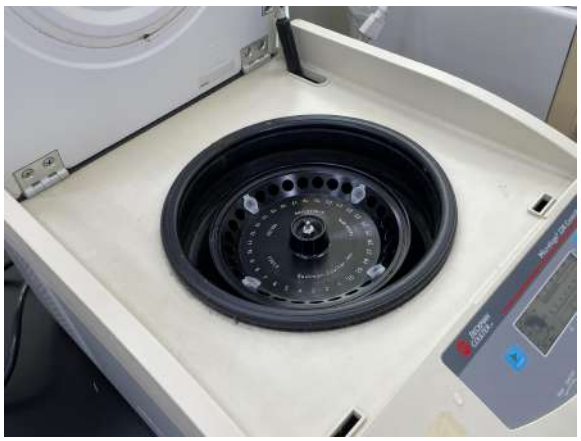



Day 2 (2024/07/02)

PCR amplification

1. Using the cDNA that we had obtained previously, we transferred 2μL of cDNA into a new Eppendorf. Added 10μL of PCR buffer, 2μL of dNTPs, 2μL of forward primer, 2μL of reverse primer, 0.5μL Taq polymerase and 81.6μL of ddH2O.
    a. PCR buffer provides a stable and optimal environment for the polymerase enzymes to work efficiently
    b. dNTPs is used to provide the corresponding nucleotides to the cDNA template strand.
    c. forward and reverse primer provides a starting point for DNA synthesis by binding to the target DNA sequence
    d. Taq polymerase (enzyme) catalyzes the amplification of the DNA synthesis
    e. ddH2O (double distillation processed) used for dilution
2. Initiated PCR thermal cycling
3. initial denaturation at 94°C for 30 seconds
4. performed 34 cycles of:
    a. Denaturation at 94°C for 30 seconds
    b. annealing at 53°C for 30 seconds
    c. elongation at 68°C for 90 seconds
5. performed a final elongation at 68°C for 10 minutes and then allowed it to be cool down under 4°C
6. at the end of the PCR we successfully cloned our target DNA from the cDNA template.

electrophoresis process to separate the HSP70 gene fragment from others

1. Combined TAE buffer and agarose powder (0.25g of agarose in 25mL TAE) and microwaved the solution until boiling with bubbles
2. incubated in 60°C water bath for 5 minutes and poured into a gel tray with a well comb to solidify and form the gel

3. transferred the gel into a container and added 100mL of TAE buffer and 10µL of SYBR gold to stain DNA for visualization under UV light and placed it on a shaker at 50 RPM for 30 minutes
4. added TAE buffer to the electrophoresis apparatus to completely submerge the gel that was placed in the apparatus
5. Added DNA loading dye into the DNA sample
6. Transferred the DNA sample into a new Eppendorf, centrifuged and then loaded it into the gel wells
    a. we had six wells therefore for the one on the very left, we added 1kb DNA marker as a standard and filled the rest of the wells with the DNA sample
7. ran the electrophoresis under 100V. Observation: the dye moved away from the wells which could be identified by the presence of the loading dye
8. observed the movement of DNA on a UV cabinet. Observation:one clear band
9. Used a scalpel and cut the band of DNA that aligned with the standard off and then placed into a new Eppendorf
10. Added 3x the volume of the gel of QXI into the eppendorf
11. vortexed it, added 10µL of QIAEX II as it helps melt the agarose gel
12. incubated in 50°C water bath for 15 minutes. (Every 2 minutes, took out the Eppendorf and vortexed it for 10 seconds)
13. centrifuged under 18,000 x g for 30 seconds and discarded all supernatant
14. added 500µL QXI and vortexed it
15. centrifuged under 18,000 x g for 30 seconds and discarded all supernatant
16. added 500µL Buffer PE and vortexed it
17. centrifuged under 18,000 x g for 30 seconds and discarded all supernatant
18. Repeat step 15 and 16
19. let it dry for 15 minutes until pellet turned milk white
20. added 10µL ddH2O, vortexed it, and incubated in 50°C water bath for 5 minutes
21. centrifuged under 18,000 x g for 30 seconds and transfer the supernatant into a new Eppendorf
22. Used a nanodrop to measure the concentration as well as the quality of the collected DNA sample
    a. Cleaned the nanodrop machine platform with Kimwipes
    b. Added 2µL ddH2O to the probe platform and closed the probe
    c. Opened the software, selected “Nucleic acid” mode, made sure the “sample type” is “DNA.”
    d. Selected “Blank” to initialize
    e. Cleaned the nanodrop machine platform with Kimwipes
    f. Added 2µL purified DNA sample to the probe platform and closed the probe
    g. Selected “Measure” to evaluate the Concentration (ng/µL) and Absorbance (A260/A230 and A260/A280), noted and took pictures of the data results
    h. Cleaned the nanodrop machine platform with Kimwipes
    i. Added 2µL ddH2O to the probe platform and closed the probe
    j. Cleaned the nanodrop machine platform with Kimwipes
    k. Closed the software, computer, and nanodrop

23. Stored the DNA samples under -20°C

Data
concentration: 8.65 ng/μL
A260/280: 1.37
A260/230: 0.535

Day 3 (2024/07/03)
Ligation Reaction

1. Thawed the sample on ice
2. Added 1μL 10X Ligase Buffer, 2μL T&A™ Cloning Vector, 6μL Purified PCR product, and 1μL T4 DNA Ligase (last added) to a new Eppendorf
3. Mixed the sample by pipetting
4. Placed the sample under room temperature (15-25°C) for 2 hours

Transformation

5. Thawedthe *E. coli* DH5α on ice
6. Lighted the alcohol lamp to prevent from contamination
7. Added 50μL *E.coli* DH5α to a new Eppendorf
8. Added 10μL of the Ligation Reaction sample to this Eppendorf
9. Mixed the sample by pipetting
10. Placed the Eppendorf on ice for 30 minutes
11. Transferred the sample to a 42°C dry bath for 90 seconds
12. Placed the sample on ice for 5 minutes
13. Added 200μL SOC medium to the sample
14. Placed the sample to a 37°C Incubator for 1 hour
15. Added 5-8 glass beads to the Amp+ LB medium
16. Added 87μL of the sample to the Amp+ LB medium
17. Closed the cap of the Amp+ LB medium
18. Shook the Amp+ LB medium to spread the sample evenly among the medium surface until it is shown to be visibly dry on the surface
19. Removed the glass beads
20. Placed the Amp+ LB medium to a 37°C Incubator for 14-16 hours

Day 4 (2024/07/04)
Colony PCR

1. After letting the agarose incubate at 37°C for 16 hours, white tiny spots were observed scattered around the plate.
2. Took a new Eppendorf added 30µL of PCR master mix, 6µL of forward primer, 6µL of reverse primer and 18µL of ddH2O and mixed well with a pipette
3. Distributed the solution across five tiny Eppendorf (10µL volume each)
4. Used the plastic tip of an Eppendorf and selected 5 white spots on the agarose plate and scooped them up individually and transferred them into each tiny Eppendorf
5. initial denaturation at 94°C for 10 minutes
6. performed **30** cycles of:
    a. Denaturation at 94°C for 30 seconds
    b. annealing at 55°C for 30 seconds
    c. elongation at 72°C for 90 seconds
7. performed a final elongation at 72°C for 10 minutes and then allowed it to be cool down under 4°C
8. Combined TAE buffer and agarose powder (0.25g of agarose in 25mL TAE) and microwaved the solution until boiling with bubbles
9. incubated in 60°C water bath for 5 minutes and poured into a gel tray with a well comb to solidify and form the gel
10. transferred the gel into a container and added 100mL of TAE buffer and 10µL of sybr gold to stain DNA for visualization under UV light and placed it on a shaker at 50 RPM for 30 minutes
11. added TAE buffer to the electrophoresis apparatus to completely submerge the gel that was placed in the apparatus
12. Transferred the DNA sample into a new Eppendorf, centrifuged and then loaded it into the gel wells
    a. we had six wells therefore for the one on the very right, we added 1kb DNA marker as a standard and filled the rest of the wells with the DNA sample
13. ran the electrophoresis under 110V for 30 minutes. Observation: the dye moved away from the wells which could be identified by the presence of the loading dye
    a. Targeted length: 800-900 bp for Housekeeping gene
14. Inoculate the positive clones into 2 mL Amp+ LB liquid culture and incubated under 37°C at 200 rpm shake for 14-16 hours

Lab Notes Experiments List

- **[Formosa_101] [20240713] pET-9a-PibpA-HSP70- His-T2A-RFP, and pET-3a-PcspA-otsA-Flag-T2A- otsB-Myc-T2A-EGFP Plasmid Midi Prep**
- **[Formosa_101] [20240724] Co-Transformation Tests**
- **[Formosa_101] [20240726] Target Protein Expression (Pre-test)**
- **[Formosa_101] [20240815] Target Protein Expression**
- **[Formosa_101] [20240828] Target Protein Expression**

[Formosa_101] [20240713] pET-9a-PibpA-HSP70-His-T2A-RFP, and pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP Plasmid Midi Prep

Purpose: Plasmid midi preparation of "pET-9a-Flag-ASR" & "pET-17b-PcpcB-LK-6XHis" & "pET-9a-PibpA-HSP70-His-T2A-RFP" & "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP"

240713

P1: Transformation

M1: competent cell E. coli strain Stbl3 (commercial)

M2: LB Kana+ agar plate

M3: LB Amp+ agar plate

M4: SOB medium

M5: pET-9a-PibpA-HSP70-His-T2A-RFP (100ng/μL)

M6: pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP (100ng/μL)

S1: Add 1μL plasmid into 30μL Stbl3.

S2: Place on ice for 10min.

S3: Place in 42℃ for 90s.

S4: Place on ice for 5min.

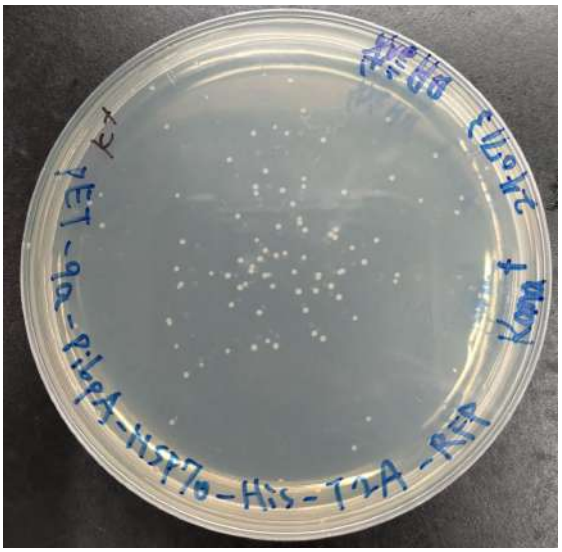
S5: Add 200 μL SOB.

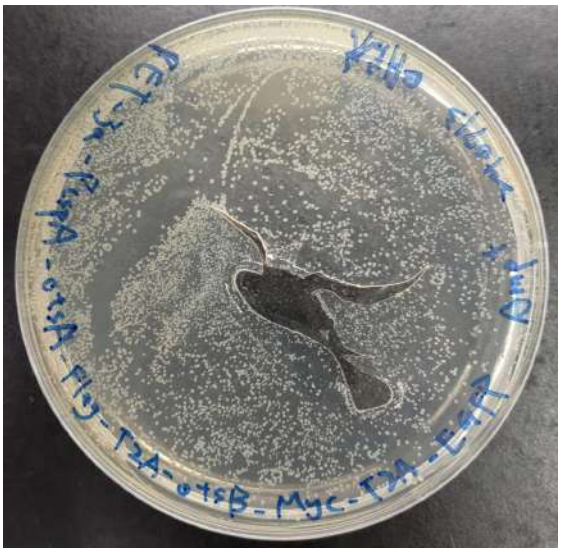
S6: Spreading an agar plate.

S7: Incubate at 37 ℃ overnight.

240714

R1: pHot & pCold plasmids.





P2: Plasmid extraction (Midi-prep)

M1: LB broth

M2: Kanamycin (50mg/mL)

M3: Ampicillin (100mg/mL)

M4: Colony from P1

M5: RES buffer (MACHEREY-NAGEL)

M6: LYS buffer (MACHEREY-NAGEL)

M7: NEU buffer (MACHEREY-NAGEL)

M8: EQU buffer (MACHEREY-NAGEL)

M9: WASH buffer (MACHEREY-NAGEL)

M10: ELU buffer (MACHEREY-NAGEL)

M11: Isopropanol

M12: 70% EtOH

M13: TE-8.0 buffer

S1: Add 2mL LB broth into a 15mL tube.

S2: Add 2μL Kanamycin or Ampicillin.

S3: Drop the tip with the colony into a 15mL tube.

S4: Incubate at 37℃ for 4hr.

S5: Add 100mL LB broth into an Erlenmeyer flask.

S6: Add 100μL Kana or Ampicillin.

S7: Transfer 100μL of bacteria solution into Erlenmeyer flask.

S8: Incubate at 37 ℃ for 16hr.

240715

S9: Pour the bacteria into a 50mL tube. Centrifuge at 6000g for 15min. Repeat the step until all the bacteria is precipitated.

S10: Add 12mL RES buffer. Resuspend completely by vortex.

S11: Add 12mL LYS buffer. Gently invert the tubes 5 times. DO NOT VORTEX. Rest in RT for 5min.

S12: Set the NucleoBond Xtra column and the filter. Add 12ml EQU buffer to rinse the filter.

S13: Add 12mL NEU buffer. Invert until the color changes from blue to white.

S14: Load the mixture into the column.

S15: Add 15mL EQU buffer to wash the filter. (1st wash)

S16: Remove the filter. (Only column left)

S17: Add 20ml WASH buffer to the column. (2nd wash)

S18: After the WASH buffer is completely flowing through, add 8mL ELU buffer. Collect the eluate in a 15mL tube.

S19: Add 5.6mL isopropanol. Vortex thoroughly. Centrifuge at 9000rpm for 30min.

S20: Discard the supernatant. Transfer the pellet in the bottom of the tube to Eppendorf. Add 1mL 70% EtOH. Centrifuge at 14000g for 5min. Discard the supernatant and add 70% EtOH. Centrifuge again. Discard the flow-through.

S21: Dry in the air.

S22: Add 100μL TE buffer.

S23: Measure the concentration of DNA. Adjust the concentration to 100ng/μL.

R1: The concentration of all the plasmids was not high enough. Thus adjust all of them to 100ng/μL.

R2: The low yield was probably because of the low copy number feature of the plasmid

240729

P3: Sequencing

M1: plasmids from P2 (100ng/μL)

M2: T7_terminator_R primer (10μM) (gctagttattgctcagcgg)

M3: HSP70_F1_Seq primer (10μM) (catcgtagccgatgaggacg)

M4: HSP70_F2_Seq primer (10μM) (gcagtagcgccgaagccagt)

M5: pCold_F primer (10μM) (ACGCCATATCGCCGAAAGG)

M6: pCold_R primer (10μM) (GGCAGGGATCTTAGATTCTG)

M7: otsA_R_Seq (10μM) (gaccttcaccgctgccttta)

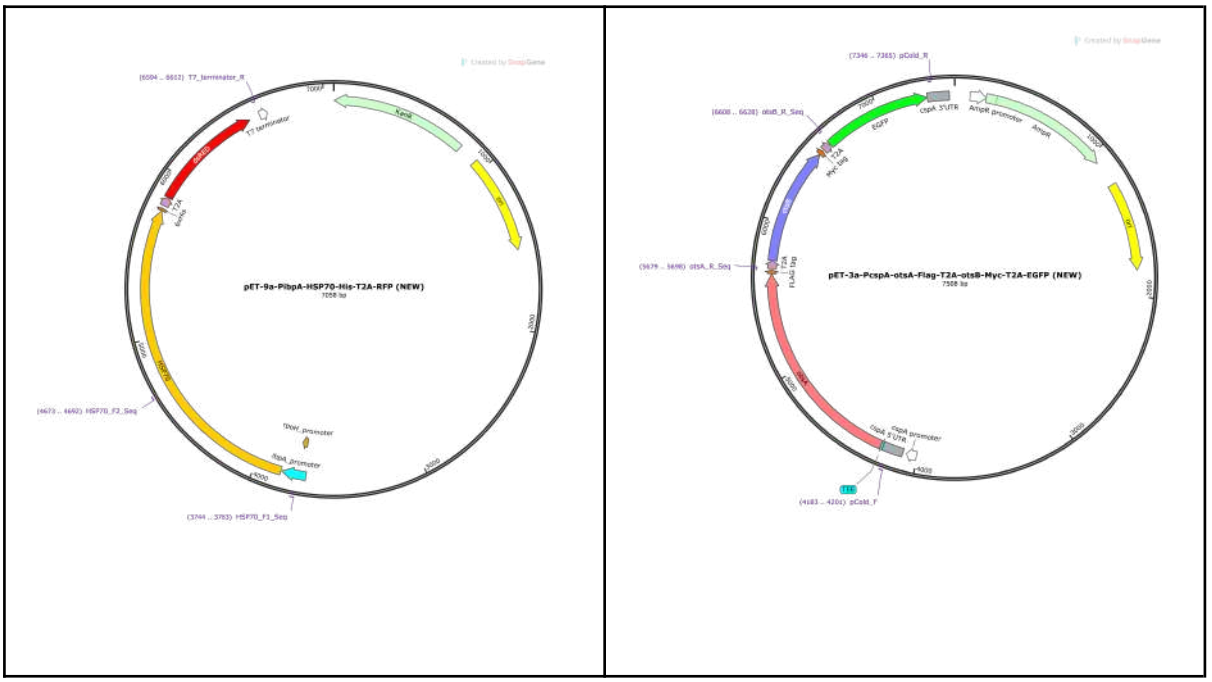
M8: otsB_R_Seq (10μM) (cttcacctttgctaaccgggc)

M9: ddH2O

S1: Mix 5μL plasmid, 1μL primer, and 8μL ddH2O.

S2: Sequencing.

R1: Sequence correct.



[Formosa_101] [20240724] Co-Transformation Test

Purpose: To test double antibiotic selection

240724

P1: Transformation

M1: competent cell E. coli strain Stbl3 (commercial)

M2: LB Amp+ agar plate

M3: LB Kana+ agar plate

M4: LB Amp+/Kana+ plate

M5: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M6: plasmid "pET-3a-PcspA-OtsA-Flag-T2A-OtsB-Myc-T2A-EGFP" (100ng/μL) (pCold)

M7: SOB medium

S1: Add 3μL of each plasmid into 75μL Stbl3 as described below.

S2: Place on ice for 30min.

S3: Place at 42℃ for 45s.

S4: Place on ice for 2min.

S5: Add 600μL SOB.

S6: Incubate at 37℃ at 100 rpm for 1hr.

S7: Spread 200μL of each transformant on an Amp+, a Kana+, and an Amp+/Kana+ agar plate.

S8: Incubate at 37 ℃ overnight.

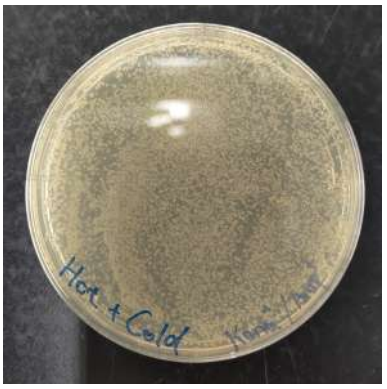
	Hot+Cold	Hot	Cold	No plasmid
Stbl3	75	75	75	75
pHot	3	3	-	-
pCold	3	-	3	-
TE buffer	-	3	3	6

Total	81	81	81	81

240725

R1: pHot contains Kanamycin resistance gene, while pCold contains Ampicillin resistance gene.

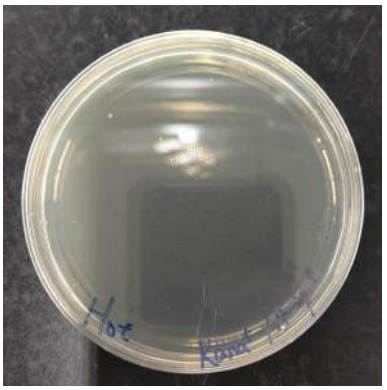
R2:



pHot/pCold - Kana+/Amp+

pHot/pCold - Kana+

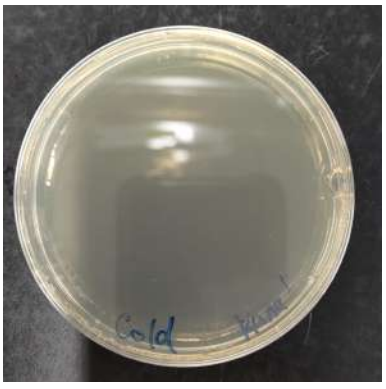
pHot/pCold - Amp+



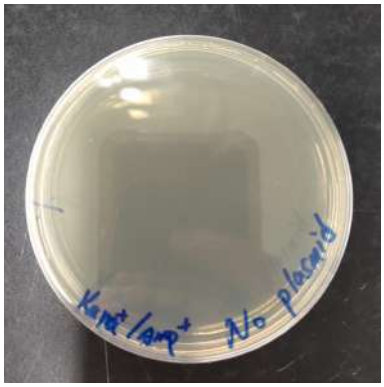
pHot- Kana+/Amp+

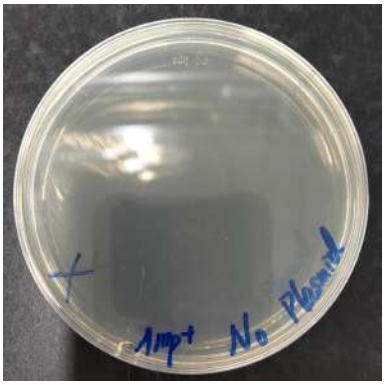
pHot - Kana+

pHot - Amp+



pCold - Kana+/Amp+ **pCold - Kana+** **pCold - Amp+**





No plasmid - Kana+/Amp+ **No plasmid - Kana+** **No plasmid - Amp+**

R3: Despite some colonies appearing on Amp+ agar plate with no AmpR plasmid transformant, the results indicated that the plasmid possessed the correct antibiotic resistance gene.

[Formosa_101] [20240726] Target Protein Expression (Pre-test)

P: To express target protein from plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" & "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" using E. coli BL21 STAR (DE3)

240724

P1: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: LB Amp+/Kana+ plate

M3: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M4: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M5: SOB medium

S1: Add 1μL of both two plasmids into 50μL BL21 STAR (DE3).

S2: Place on ice for 30min.

S3: Place at 42℃ for 45s.

S4: Place on ice for 2min.

S5: Add 1000μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

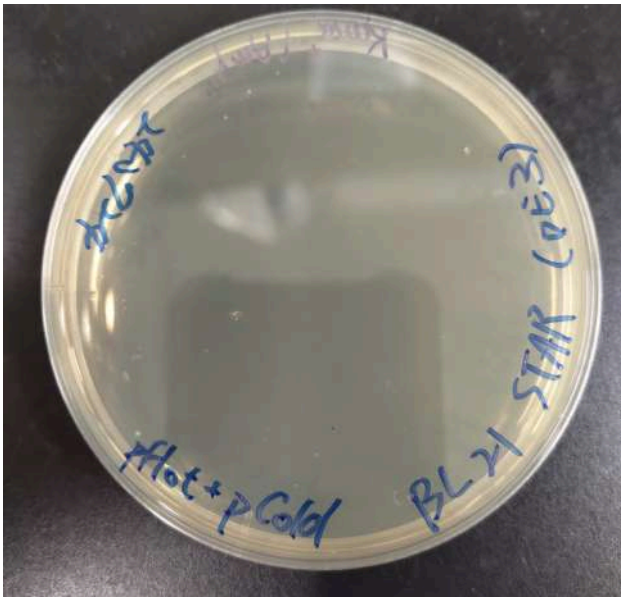
S7: Centrifuge at 6000g for 5min. Discard the supernatant and resuspend the pellet by 200μL SOB. Spread the transformant on an Amp+/Kana+ agar plate.

S8: Incubate at 37 ℃ overnight.

240725

R1: No colony. Repeat the experiment again.

R2:



P2: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: LB Amp+/Kana+ plate

M3: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M4: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M5: SOB medium

S1: Add 1μL of both plasmids DNA into 50μL BL21 STAR (DE3).

S2: Place on ice for 30min.

S3: Place at 42℃ for 45s.

S4: Place on ice for 2min.

S5: Add 1000μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Centrifuge at 6000g for 5min. Discard the supernatant and resuspend the pellet by 200μL SOB. Spread the transformant on an Amp+/Kana+ agar plate.

S8: Incubate at 37 ℃ overnight.

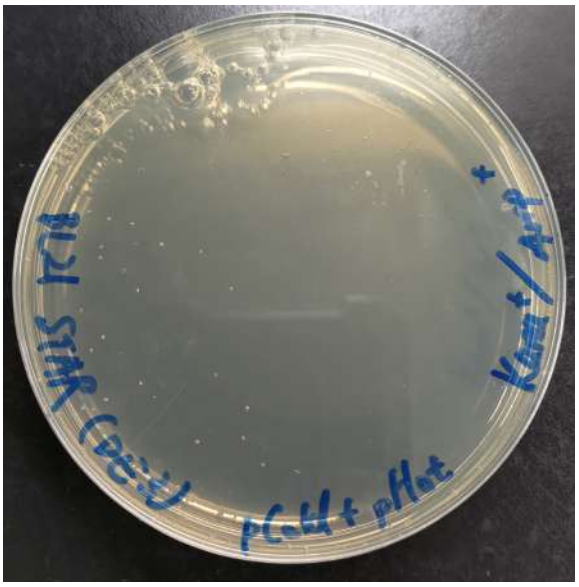
240726

R1: Still no colony. Repeat the experiment again.

R2: The white dots on the photo were bubbles.

R3: Extending heat shock time to 90s might increase the efficiency of transformation.

R4:



P3: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: competent cell *E. coli* strain BL21

M3: LB Amp+/Kana+ plate

M4: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M5: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M6: SOB medium

S1: Add 1μL of both two plasmids into 50μL BL21 STAR (DE3) or BL21.

S2: Place on ice for 30min.

S3: Place at 42℃ for 90s.

S4: Place on ice for 2min.

S5: Add 200μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Spread the transformant on an Amp+/Kana+ agar plate.

S8: Incubate at 37 ℃ overnight.

240727

R1: BL21 strain was also used for transformation this time.

R2: Still no colony on both BL21 and BL21 STAR (DE3) plates. Repeat the experiment again with lower concentration of plasmid DNA (10ng).

R3: Transformation results of BL21 STAR (DE3) & BL21.

<u>P3: Transformation</u>

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: competent cell *E. coli* strain BL21

M3: LB Amp+/Kana+ plate

M4: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M5: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M6: SOB medium

S1: Add 1μL of both two plasmids into 50μL BL21 STAR (DE3) or BL21.

S2: Place on ice for 30min.

S3: Place at 42℃ for 90s.

S4: Place on ice for 2min.

S5: Add 200μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Spread the transformant on an Amp+/Kana+ agar plate.

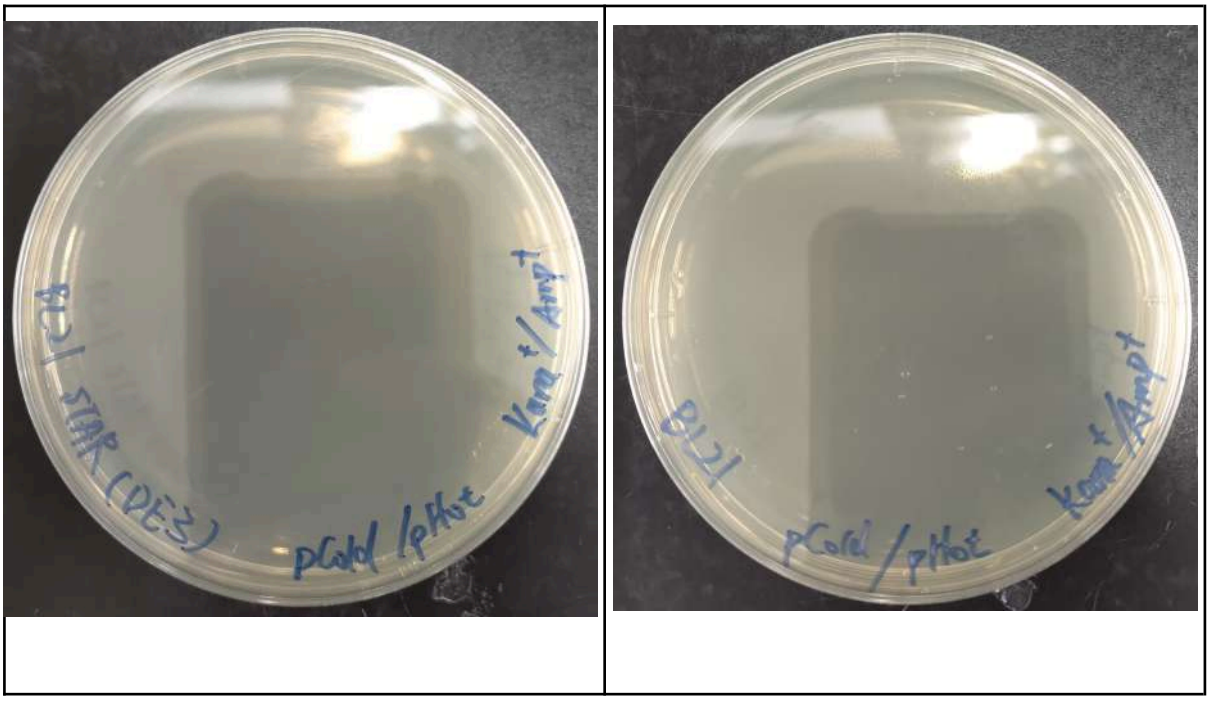
S8: Incubate at 37 ℃ overnight.

240727

R1: BL21 strain was also used for transformation this time.

R2: Still no colony on both BL21 and BL21 STAR (DE3) plates. Repeat the experiment again with lower concentration of plasmid DNA (10ng).

R3: Transformation results of BL21 STAR (DE3) & BL21.



P4: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: LB Amp+/Kana+ plate

M3: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M4: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M5: SOB medium

S1: Dilute both plasmid DNA to 10ng/μL. Add 1μL of both two plasmids into 50μL BL21 STAR (DE3).

S2: Place on ice for 30min.

S3: Place at 42℃ for 90s.

S4: Place on ice for 2min.

S5: Add 200μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Spread the transformant on an Amp+/Kana+ agar plate.

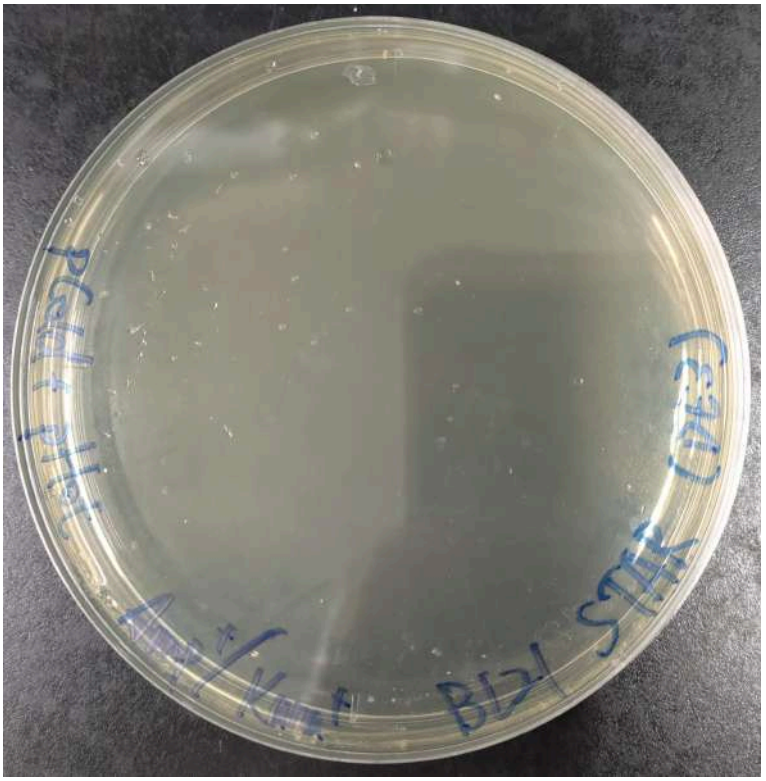
S8: Incubate at 37 ℃ overnight.

240728

R1: Still no colony. Repeat the experiment with additionally single plasmid groups.

R2: Lower the incubation temperature might help with the result. Repeat the experiment with an additionally 30℃ incubation group.

R3:



P5: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: competent cell *E. coli* strain BL21

M3: LB Amp+/Kana+ plate

M4: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M5: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M6: SOB medium

S1: Prepare 5 transformation groups. Two of them were co-transformed with pCold & pHot using BL21 STAR (DE3) strain, one was incubated at 37℃ and another was at 30℃ overnight. One of them was also co-transformed with pCold & pHot using BL21 strain instead. The rest of the two groups were transformed with pCold or pHot using BL21 STAR (DE3) strain. For each group, add 1μL of both corresponding plasmid (10ng/μL) into 50μL BL21 STAR (DE3) or BL21.

S2: Place on ice for 30min.

S3: Place at 42℃ for 90s.

S4: Place on ice for 2min.

S5: Add 200μL SOB.

S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Spread the transformant on an Amp+/Kana+ agar plate, an Amp+ agar plate, or a Kana+ plate.

S8: Incubate at 37 ℃ overnight. (Except for the 30℃ group.)

240729

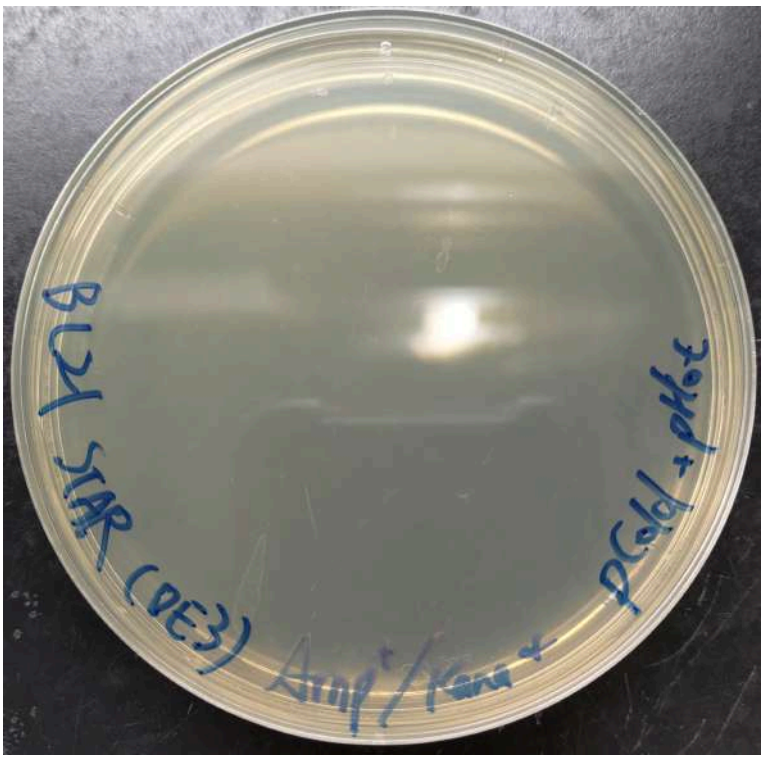
R1: The colonies grew only on single-plasmid transformation groups. Still no colonies on three co-transformation groups.

R2: Compared to abundant colonies that grew on pHot-Kana+ plate, the colonies that grew on pCold-Amp+ plate appeared to be having irregular size. The amount of colonies were fewer as well, indicating that pCold might be toxic to this BL21 STAR (DE3) strain. This situation was not observed when transformed using Stbl3 strain.

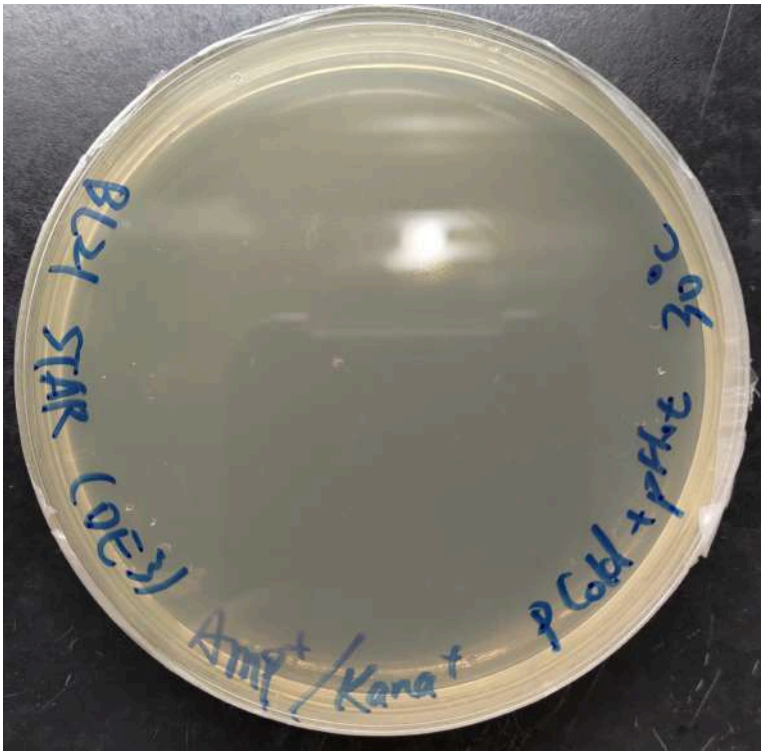
R3: To overcome this obstacle, attempt co-transformed with different plasmid DNA concentration ratio next time. (pCold:pHot = 10:1)

R4: Alternate SOB medium with SOC might help with the bacteria recovery, thus improving the transformation results.

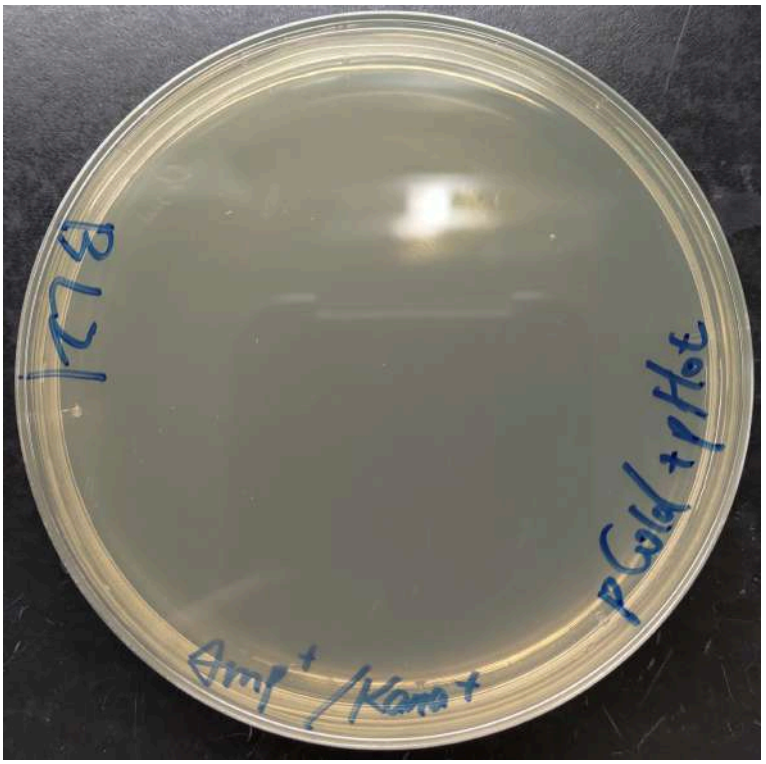
R5: pCold+pHot co-transformation using BL21 STAR (DE3), incubated at 37℃. (Amp+/Kana+)



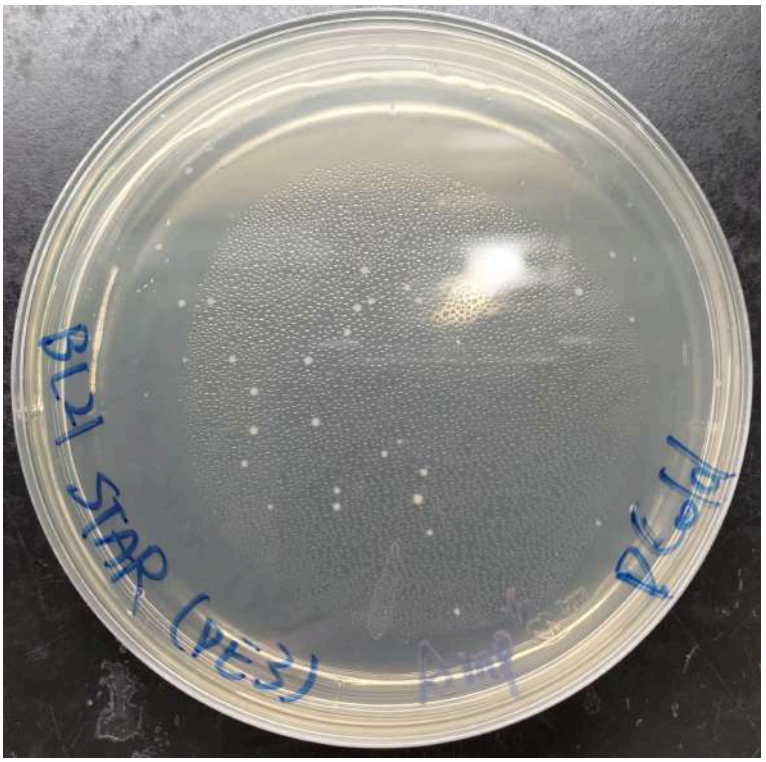
R6: pCold+pHot co-transformation using BL21 STAR (DE3), incubated at 30℃. (Amp+/Kana+)



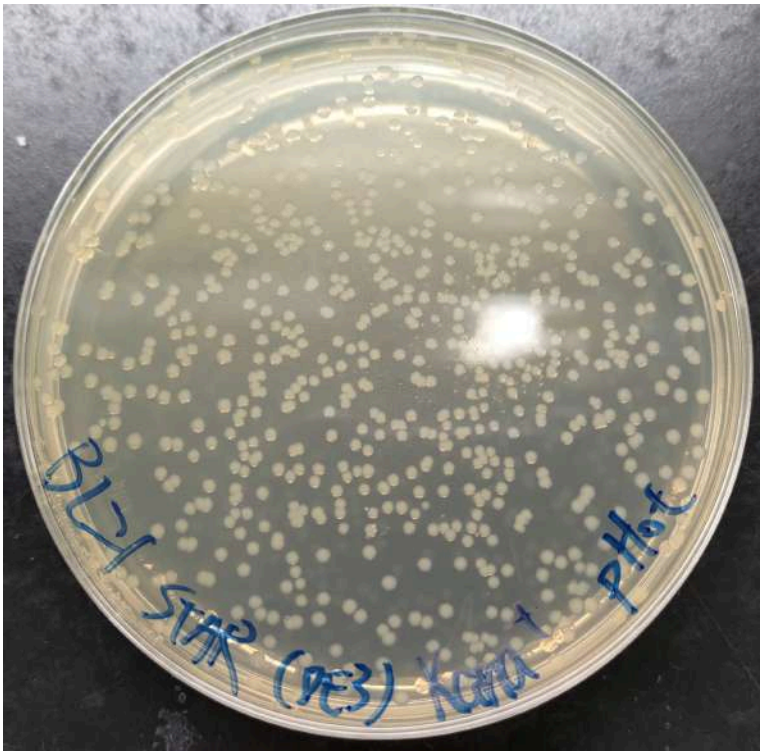
R7: pCold+pHot co-transformation using BL21. (Amp+/Kana+)



R8: pCold transformation using BL21 STAR (DE3). (Amp+)



R9: pHot transformation using BL21 STAR (DE3). (Kana+)



P6: Bacterial incubation test

M1: LB medium

M2: Ampicillin stock (100mg/mL)

M3: pCold-Amp+ agar plate from P5

S1: Add 1.5μL ampicillin stock into 1.5mL LB medium (final concentration = 100μg/mL).

S2: Inoculate a colony to the medium and incubate at 37℃ for 4hr.

S3: After 4hr, inoculate 500μL bacterial culture to a 5mL LB medium containing the same concentration of ampicillin. Incubate at 37℃ overnight.

R1: The purpose of this experiment was to determine whether the pCold plasmid was toxic to BL21 STAR (DE3) strain.

R2: Turbid bacterial culture was observed on the next day, indicating that it grew normally.

P7: Transformation

M1: competent cell *E. coli* strain BL21 STAR (DE3) (Invitrogen)

M2: LB Amp+/Kana+ plate

M3: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)

M4: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)

M5: SOC medium

S1: Dilute pHot plasmid DNA to 10ng/μL. Prepare two transformants. For each group, add 10ng of pHot and 100ng of pCold into 50μL BL21 STAR (DE3).

S2: Place on ice for 30min.

S3: Place at 42℃ for 90s.

S4: Place on ice for 2min.

S5: Add 200μL SOC.

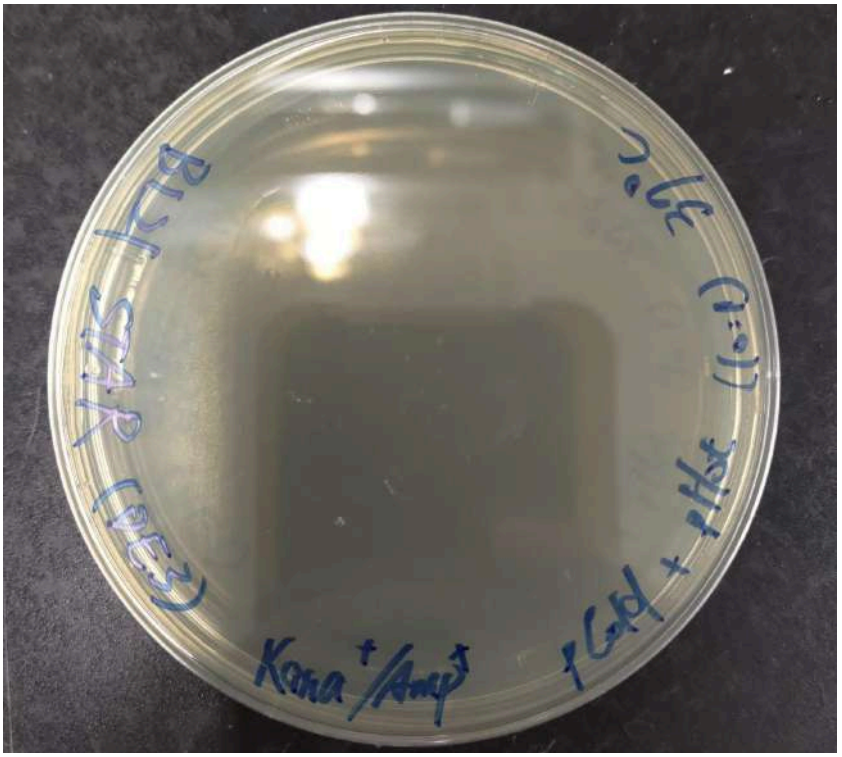
S6: Incubate at 37℃ at 100rpm for 1hr.

S7: Spread the transformant on an Amp+/Kana+ agar plate.

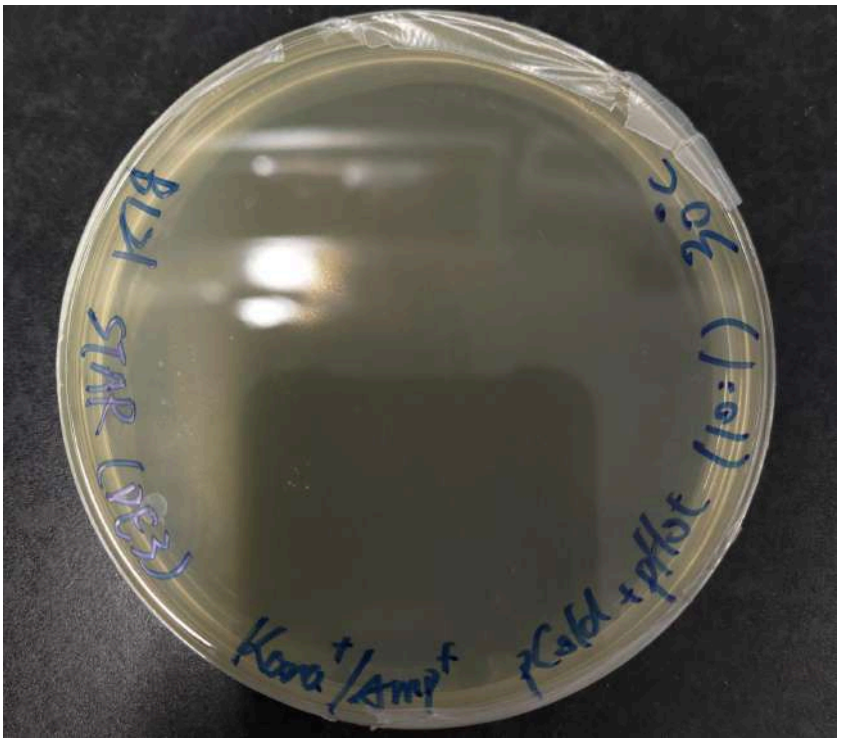
S8: Incubate one of the plates at 37℃ while another one at 30℃ overnight.

240730

R1: Fortunately, a single colony appeared on the plate incubated at 37℃.



R2: No colony on the plate incubated at 30℃.



P8: Streaking plate and preparing bacterial glycerol stock

M1: the colony on the pCold+pHot Amp+/Kana+ agar plate from P7

M2: Amp+/Kana+ agar plate

M3: LB medium

M4: Ampicillin stock (100mg/mL)

M5: Kanamycin stock (50mg/mL)

M6: 50% glycerol (sterilized)

S1: Inoculate the only colony to 1.5mL LB medium containing 50μg/mL kanamycin and 100μg/mL ampicillin. Incubate at 37℃ for 4hr.

S2: After 4hr, take 100μL bacterial culture. Centrifuge at 6000g for 5min. Discard the supernatant, dip the pellet using an inoculation loop and streak the agar plate.

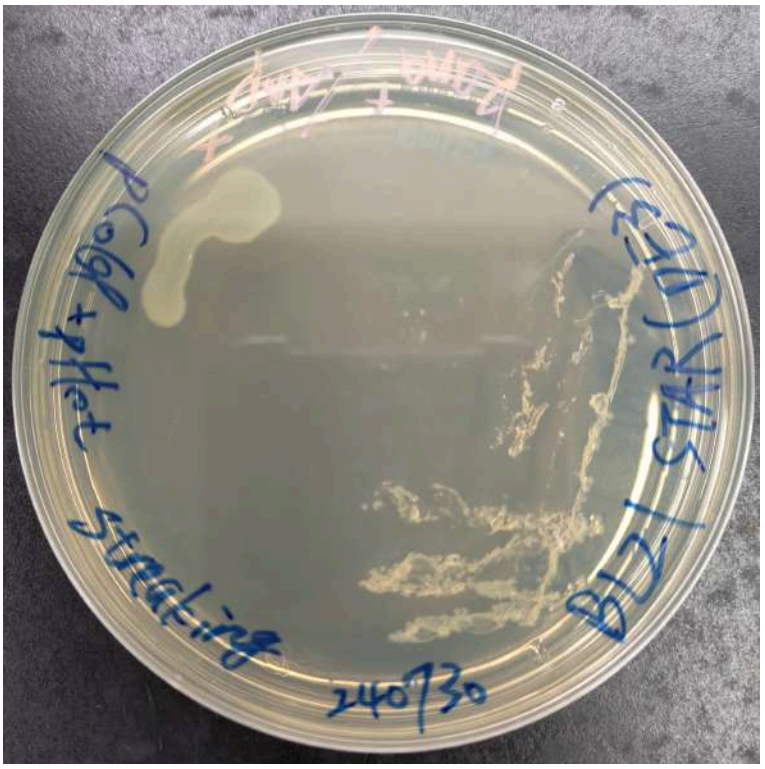
S3: Meanwhile, take another 100μL bacterial culture and directly add it onto the same plate. Avoid the streaking area. Then, incubate at 37℃ overnight.

S4: Inoculate 1mL bacterial culture to 19mL LB medium containing kanamycin and ampicillin. Incubate at 37℃ overnight.

240731

S5: Prepare 15 cryogenic vials. For each vial, mix 750μL overnight culture and 750μL glycerol stock. Freeze them at -80℃.

R1: Bacteria grew normally on the plate.



P9: Protein expression

M1: Colonies on P5 (pCold or pHot) or P8 agar plate (pCold+pHot)

M2: LB medium

M3: Ampicillin stock (100mg/mL)

M4: Kanamycin stock (50mg/mL)

M5: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)

M6: 5X sample buffer (50% glycerol, 10%SDS, 250mM Tris, 0.05% bromophenol blue, 5% β-Mercaptoethanol, pH=6.8)

M7: Protein extraction buffer (80% TBS buffer + 20% 5X sample buffer)

S1: Inoculate a colony to 1mL LB medium containing 50μg/mL Kanamycin and/or 100μg/mL Ampicillin. Incubate at 30℃ for 4hr.

S2: Inoculate 100μL culture to 10mL LB medium (Kana+/Amp+). Incubate at 30℃ overnight.

240801

S3: Measure OD600 value. Dilute each culture to OD600 value~=0.1 with LB medium (Kana+/Amp+). Incubate at 30℃ for 4hr. Then, dispense 4mL of each culture to 2 centrifuge tubes. Label them as "20℃", "42℃".

S4: Incubate at 20℃ or 42℃ for 3hr. Observe the expression of fluorescent protein.

S5: Since no significant color change was observed in the culture medium, keep incubating overnight.

240802

S6: Measure OD600 value of each culture.

S7: Harvest bacteria with the amount equals 1mL of OD600=1.0 culture by centrifuging the bacterial culture at 4℃ at 6000g for 5min. Discard the supernatant.

S8: Resuspend bacterial pellet with 1mL TBS buffer for washing. Centrifuge at 4℃ at 6000g for 5min. Discard the supernatant.

S9: Resuspend the pellet with 100μL protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer).

S10: Incubate each sample at 100℃ for 10min. After cooling down to room temperature, store them at -20℃.

R1: OD600 value of each culture at S3:

- pCold: 0.7
- pHot: 1.15
- pCold+pHot: 0.7

R2: OD600 value of each culture (42℃) at S6:

- pCold : 1.80
- pHot: 1.87
- pCold+pHot: 1.78

R3: OD600 value of each culture (20℃) at S6:

- pCold : 1.57
- pHot: 1.62
- pCold+pHot: 1.59

R4: To obtain quantitative bacterial protein samples, about 1.0 = 8 x 10^8 bacteria cells (~1mL of OD600=1.0 bacterial culture) were harvested with 100μL protein extraction buffer.
R5: No significant GFP or RFP were observed on bacterial pellets. Repeat the induction experiment again. However, we still store protein samples for western blotting.

P10: Protein expression

M1: Colonies on P5 (pCold or pHot) or P8 agar plate (pCold+pHot)
M2: LB medium
M3: Ampicillin stock (100mg/mL)
M4: Kanamycin stock (50mg/mL)
M5: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)
M6: 5X sample buffer (50% glycerol, 10%SDS, 250mM Tris, 0.05% bromophenol blue, 5% β-Mercaptoethanol, pH=6.8)
M7: Protein extraction buffer (80% TBS buffer + 20% 5X sample buffer)

S1: Inoculate a colony to 10mL LB medium containing 50μg/mL Kanamycin and/or 100μg/mL Ampicillin. Incubate at 30℃ overnight.

240803

S2: Measure OD600 value. Dilute each culture to OD600 value~=0.1 with LB medium (Kana+/Amp+). Incubate at 30℃ for 3hr. Then, dispense 5mL of each culture to 3 centrifuge tubes. Label them as "15℃", "30℃", "42℃".
S3: Incubate at 30℃ for 4hr.
S4: Measure OD600 value of each culture.
S5: Harvest bacteria with the amount equals 1mL of OD600=1.0 culture by centrifuging the bacterial culture at 4℃ at 6000g for 5min. Discard the supernatant.
S6: Resuspend bacterial pellet with 1mL TBS buffer for washing. Centrifuge at 4℃ at 6000g for 5min. Discard the supernatant.
S7: Resuspend the pellet with 100μL protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer).

S8: Incubate each sample at 100℃ for 10min. After cooling down to room temperature, store them at -20℃.

R1: OD600 value of each culture at S2:

- pCold: 0.75
- pHot: 1.10
- pCold+pHot: 0.85

R2: OD600 value of each culture (42℃) at S4:

- pCold : 1.81
- pHot: 1.71
- pCold+pHot: 1.63

R3: OD600 value of each culture (30℃) at S4:

- pCold : 1.36
- pHot: 1.43
- pCold+pHot: 1.05

R4: OD600 value of each culture (15℃) at S4:

- pCold : 0.99
- pHot: 1.19
- pCold+pHot: 0.77

R5: To obtain quantitative bacterial protein samples, about 1.0 = 8 x 10^8 bacteria cells (~1mL of OD600=1.0 bacterial culture) were harvested with 100μL protein extraction buffer.

R6: Still no significant GFP or RFP were observed on bacterial pellets. However, it was probably because the expressions were not high enough to be seen by naked eyes. Observe the bacteria using a fluorescent microscope next time.

240806

P11: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P10

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up 2 electrophoresis chambers. One for western blotting and one for CBR staining.

S2: Prepare 15mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 5mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P10. Load 10µL of each sample to each well. Load 10µL pre-stained protein markers as well.

S9: Run the electrophoresis at 40mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

R1: Failed to conduct SDS-PAGE experiment due to abnormal samples. The protein samples were too sticky and unable to be loaded to the bottom in wells. This is probably because of underestimated bacterial concentration while harvesting. Excessive lipids that dissolved in the buffer causing it to be sticky.
R2: Repeat the bacterial culturing and target protein expression experiments again.
R3: On the other hand, to verify whether the bacteria contain both plasmids, colony PCR experiments should be conducted in advance.

P12: Colony PCR

M1: bacterial glycerol stock from P8
M2: plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" (100ng/μL) (pHot)
M3: plasmid "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" (100g/μL) (pCold)
M4: ddH2O
M5: T7_terminator_R primer (10μM) (gctagttattgctcagcgg)
M6: HSP70_F1_Seq primer (10μM) (catcgtagccgatgaggacg)
M7: HSP70_F2_Seq primer (10μM) (gcagtagcgccgaagccagt)
M8: pCold_F primer (10μM) (ACGCCATATCGCCGAAAGG)
M9: pCold_R primer (10μM) (GGCAGGGATCTTAGATTCTG)
M10: otsA_R_Seq primer (10μM) (gaccttcaccgctgccttta)
M11: otsB_R_Seq primer (10μM) (cttcacctttgctaaccgggc)
M12: 2X Ready to Load PCR Master Mix (Cyrusbio)

S1: Harvest 500μL bacteria by centrifuging the bacterial glycerol stock at 4℃ at 6000g for 5min. Discard the supernatant.
S2: Prepare solution as described below (PCR tubes*3). Two plasmids samples are used as positive control.
S3: Dip the pellet with a tip to PCR mixtures.
S4: Run PCR (95℃ for 10min → **[**95℃ for 30s → 52℃ for 30s → 72℃ for 1min**]** *25 cycles, → 72℃ for 10min → 4℃ ∞)

Materials	Sample (μL)									
	E. coli					pHot (positive control)		pCold (positive control)		
	#1	#2	#3	#4	#5	H-1	H-2	C-1	C-2	C-3
Bacterial pellet	+	+	+	+	+	-	-	-	-	-
Plasmid pHot	0	0	0	0	0	1	1	0	0	0
Plasmid pCold	0	0	0	0	0	0	0	1	1	1
HSP70_F1_Seq primer (10μM)	1	0	0	0	0	1	0	0	0	0
HSP70_F2_Seq primer (10μM)	0	1	0	0	0	0	1	0	0	0
T7_R primer (10μM)	1	1	0	0	0	1	1	0	0	0
pCold_F primer (10μM)	0	0	1	0	0	0	0	1	1	1
otsA_R_Seq primer (10μM)	0	0	0	1	0	0	0	1	0	0
otsB_R_Seq primer (10μM)	0	0	0	0	1	0	0	0	1	0
pCold_R primer (10μM)	0	0	1	1	1	0	0	0	0	1
2X Ready to load PCR Master Mix	5	5	5	5	5	5	5	5	5	5
ddH2O	3	3	3	3	3	2	2	2	2	2
Total Volume	10	10	10	10	10	10	10	10	10	10

P13: Agarose Gel Electrophoresis

M1: Agarose

M2: 1X TAE buffer

M3: EtBr

M4: 100bb marker

M5: PCR sample from P12

S1: Add 0.5g agarose to 50mL 1X TAE buffer (1% gel).

S2: Mix and dissolve agarose in the microwave.

S3: Add 5μL EtBr (1/10000).

S4: Pour the melted agar into a gel tray with a well comb.

S5: Cool down at room temperature.

S6: Place the gel in gel box

S7: Add 6μL 100bp marker.

S8: Add PCR samples into wells.

S9: Start electrophoresis for about 15~20min.

S10: Light the gel with UV.

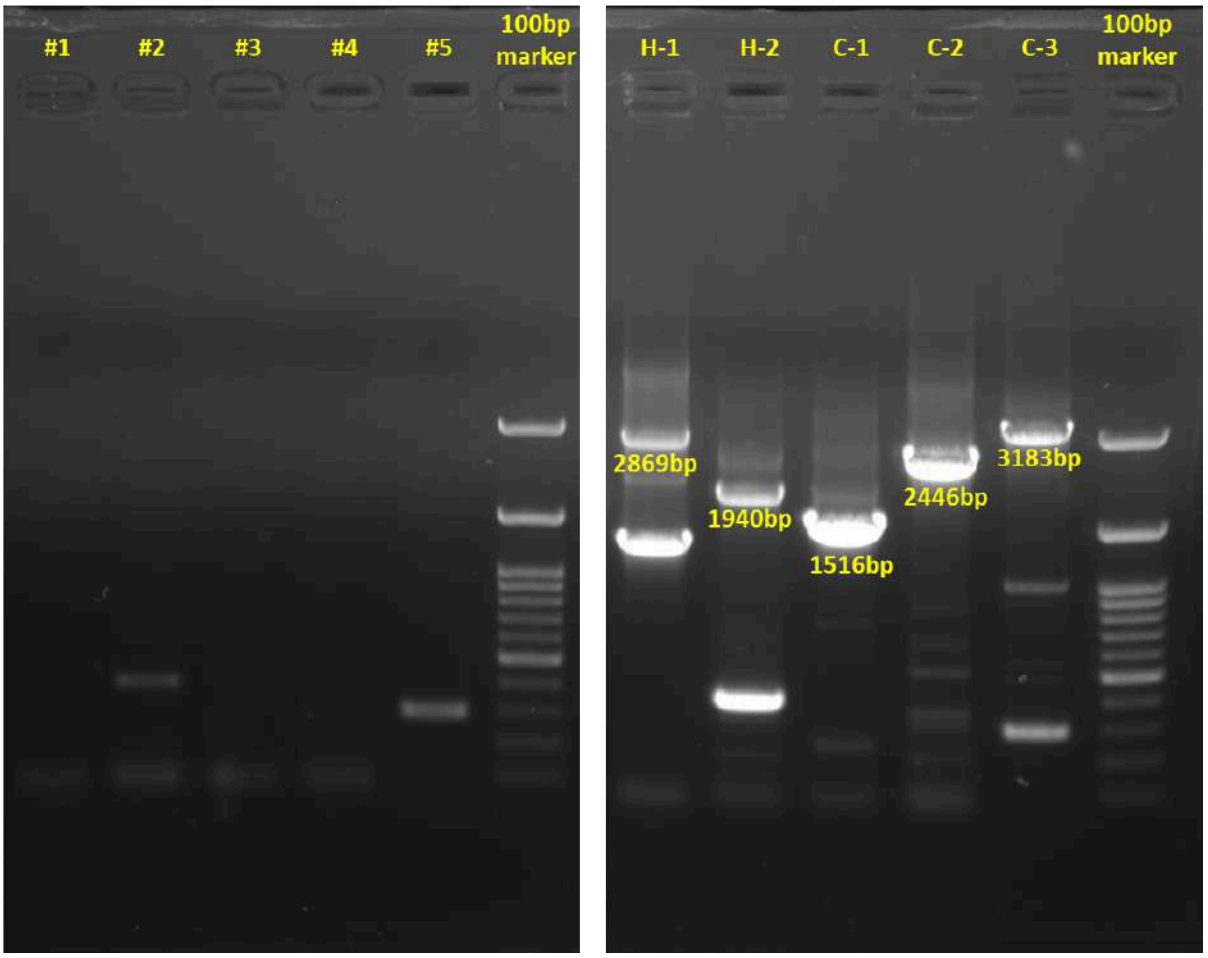
R1: Colony PCR experiments were conducted in order to verify whether the *E. coli* in glycerol stock contains both plasmids. In addition, normal PCR using plasmids themselves as DNA templates were also conducted as positive control.

R2: PCR results. (Left: *E. coli* group; Right: positive control groups)

R3: No correct band was observed in the *E. coli* group, indicating that plasmids were not retained in cells.

R4: Some unknown bands were observed. It was probably because of off-target primer bindings.

R5: Pick colonies from agar plate (P8) and repeat colony PCR again. Expand and store *E. coli* that contains both plasmids correctly.



P14: Colony PCR

M1: bacterial glycerol stock from P12

M2: Colonies on pHot/pCold Amp+/Kana+ agar plate from P8

M3: LB medium

M4: Ampicillin stock (100mg/mL)

M5: Kanamycin stock (50mg/mL)

M6: T7_terminator_R primer (10µM) (gctagttattgctcagcgg)

M7: HSP70_F1_Seq primer (10µM) (catcgtagccgatgaggacg)

M8: HSP70_F2_Seq primer (10µM) (gcagtagcgccgaagccagt)

M9: pCold_F primer (10µM) (ACGCCATATCGCCGAAAGG)

M10: pCold_R primer (10μM) (GGCAGGGATCTTAGATTCTG)

M11: otsA_R_Seq primer (10μM) (gaccttcaccgctgccttta)

M12: otsB_R_Seq primer (10μM) (cttcacctttgctaaccgggc)

M13: 2X Ready to Load PCR Master Mix (Cyrusbio)

M14: ddH2O

S1: Inoculate the rest of the bacterial glycerol stock from P12 to 29mL LB medium containing 50μg/mL Kanamycin and 100μg/mL Ampicillin. Incubate at 30℃ overnight.

240807

S2: Harvest 500μL bacteria by centrifuging the overnight culture at 4℃ at 6000g for 5min. Discard the supernatant.

S3: Prepare solution as described below (PCR tubes*34). Label them as #H0~#16 & #C0~#C16.

S4: Prepare 32 centrifuge tubes. For each centrifuge tube, add 2mL of LB medium containing 50μg/mL Kanamycin and 100μg/mL Ampicillin. Label them as sample #1~#16.

S5: Dip the pellet with a tip to PCR mixtures in PCR tube #H0 & #C0.

S6: Choose 16 colonies on the agar plate and label them as #1~#16. Dip 16 colonies with a tip and pipetting in PCR mixtures in the corresponding PCR tubes (#Hx & #Cx). Then, directly pipetting in LB medium in the corresponding 15mL centrifuge tube.

S7: Run PCR

(95℃ for 10min → **[**95℃ for 30s → 52℃ for 30s → 72℃ for 1min**]** *25 cycles, → 72℃ for 10min → 4℃ ∞)

S8: Incubate the medium in 15mL centrifuge tubes at 37℃ for 4hr.

Materials	Sample (μL)			
	#H0	#H1-H16	#C0	#C1-C16
Bacterial pellet	+		+	
Colony (#1 ~ #16)		+		+
HSP70_F1_Seq primer (10μM)	1	1	0	0
T7_R primer (10μM)	1	1	0	0
pCold_F primer (10μM)	0	0	1	1
otsA_R_Seq primer (10μM)	0	0	1	1
2X Ready to load PCR Master Mix	5	5	5	5
ddH2O	3	3	3	3
Total Volume	10	10	10	10

P15: Agarose Gel Electrophoresis

M1: Agarose

M2: 1X TAE buffer

M3: EtBr

M4: 100bp marker

M5: PCR sample from P14

S1: Add 0.5g agarose to 50mL 1X TAE buffer (1% gel).

S2: Mix and dissolve agarose in the microwave.

S3: Add 5μL EtBr (1/10000).

S4: Pour the melted agar into a gel tray with a well comb.

S5: Cool down at room temperature.

S6: Place the gel in gel box

S7: Add 6μL 100bp marker.

S8: Add PCR samples into wells.

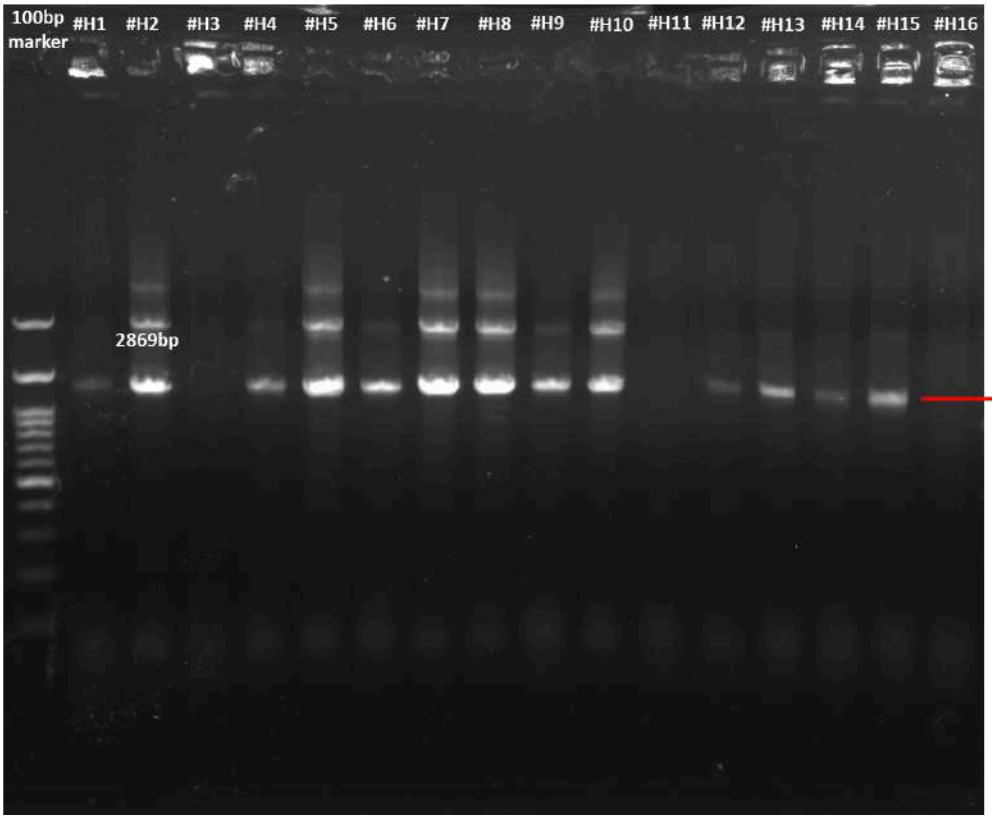
S9: Start electrophoresis for about 15~20min.

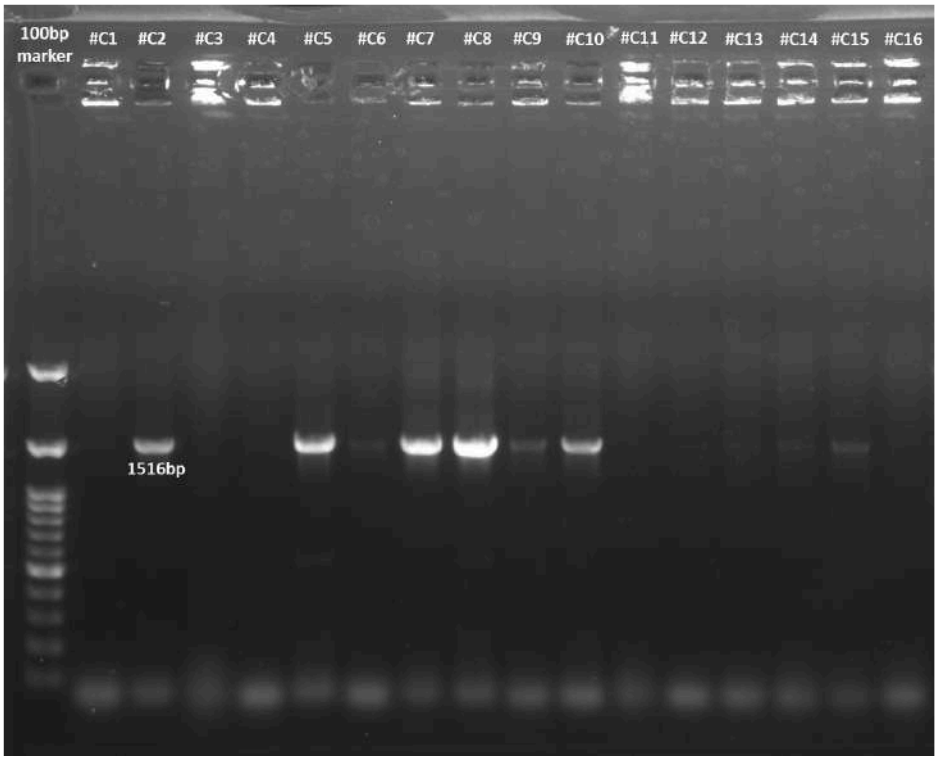
S10: Light the gel with UV.

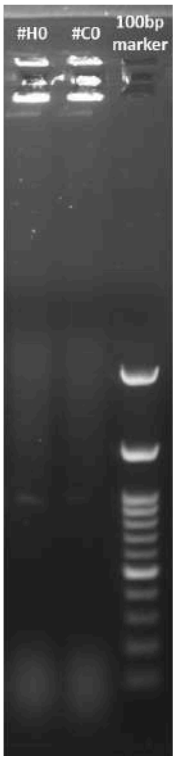
R1: Sample #H0 & #C0 were PCR products from the overnight culture. This bacterial culture originated from a bacterial glycerol stock that was used in a colony PCR experiment in P12. In the previous result, no significant band was observed on an electrophoresis gel. Same results were observed after overnight culturing, indicating that those glycerol stocks prepared in P8 should be discarded because both pHot and pCold plasmids were not retained in cells properly. Thus the glycerol stocks should be prepared again.

R2: Among #H1~#H16 & #C1~#C16 groups, correct amplicon length were found in colony **#2, #5, #7, #8, and #10** using both pHot & pCold primer pairs (2869 bp &1516 bp). However, extra unknown bands were still observed within pHot primer pairs amplicons. Despite this, we still regard these five samples as successful co-transformed colonies. Prepare new glycerol stocks from these cultures.

R3: The electrophoresis results.







P16: Preparing bacterial glycerol stock

M1: bacterial culture #2, #5, #7, #8, #10 from P14 S8
M2: Amp+/Kana+ agar plate
M3: LB medium
M4: Ampicillin stock (100mg/mL)
M5: Kanamycin stock (50mg/mL)
M6: 50% glycerol (sterilized)

S1: Inoculate 1mL of each culture to 29mL LB medium containing Kanamycin and Ampicillin. Incubate these 5 cultures at 37℃ overnight.

240808

S2: Prepare 15 cryogenic vials. Freeze 5 vials for each culture. For each vial, mix 750μL overnight culture and 750μL glycerol stock. Freeze them at -80℃.

P17: Protein expression

M1: bacterial overnight culture #2 from P16.
M2: LB medium
M3: Ampicillin stock (100mg/mL)
M4: Kanamycin stock (50mg/mL)
M5: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)
M6: 5X sample buffer (50% glycerol, 10%SDS, 250mM Tris, 0.05% bromophenol blue, 5% β-Mercaptoethanol, pH=6.8)
M7: Protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer)

S1: Measure OD600 value of the overnight culture #2. Dilute the overnight culture to OD600 value~=0.2 with fresh LB medium (Kana+/Amp+). Incubate at 30℃ for 3hr. Measure OD600 value again after incubation. Dispense the culture to 7 tubes, each contains 5mL. Label them as “0h”, “4h-15”, “4h-30”, “4h-42”, “16h-15”, “16h-30”, “4h-42”, respectively.

S2: Harvest “0h” sample.

S3: For "4h" samples, incubate at 15℃, 30℃, or 42℃ at 150 rpm for 4hr.

S4: For "16h" samples, incubate at 15℃, 30℃, or 42℃ at 150 rpm for 16hr.

S5: Measure OD600 value of each sample after culturing.

S6: Harvest bacteria with the amount equal to 1mL of OD600=1.0 culture by centrifuging the bacterial culture at 4℃ at 6000g for 5min. Harvest two tubes for each bacterial sample. Then discard the supernatant.

S7: Resuspend bacterial pellets with 1mL TBS buffer for washing. Centrifuge at 4℃ at 6000g for 5min. Discard the supernatant. Store one of the harvested bacteria of each sample at -20℃ for further fluorescent microscopy observations.

S8: Resuspend another harvested pellet of each sample with 100μL protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer).

S9: Incubate each sample at 100℃ for 10min. After cooling down to room temperature, store them at -20℃ for western blotting analysis.

R1: OD600 value of overnight culture at S1: 0.77

R2: OD600 value of the diluted culture after 3hr of incubation at S1: 0.40

R3: OD600 value of each culture after protein induction:

- 0h: 0.40
- 4h-15: 0.82
- 4h-30: 1.43
- 4h-42: 1.61
- 16h-15: 1.49
- 16h-30: 1.61
- 16h-42: 1.54

R4: To obtain quantitative bacterial protein samples, about 1.0 = 8 x 10^8 bacteria cells (~1mL of OD600=1.0 bacterial culture) were harvested with 100μL protein extraction buffer.

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P18: Fluorescent microscopy observations

M1: Harvested bacterial pellets from P17

M2: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)

S1: Resuspend each pellet with 100μL TBS. Transfer it to a 96-well plate.

S2: Observe fluorescence using fluorescent microscope.

R1: Green fluorescence was observed in 16hr groups, which showed a trend that the lower the induction temperature, the brighter it was.

R2: No red fluorescence was observed in any groups. Perform western blotting analysis first to find out the reason causing no expression of RFP.

R3: Green fluorescent intensity “16h-15” > “16h-30” > 16h-42”. (400X magnification)

240813

P19: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P17

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up 2 electrophoresis chambers. One for western blotting and one for CBR staining.

S2: Prepare 15mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 5mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P17. Load 10µL of each sample and 10µL pre-stained protein markers to both gels. Sample orders were identical on both gels: marker, "0h", "4h-15", "4h-30", "4h-42", "16h-15", "16h-30", "16h-42", marker.

S9: Run the electrophoresis at 40mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

P20: Transferring

M1: SDS-polyacrylamide gel from P19

M2: PVDF membrane

M3: methanol

M4: 1X transfer buffer (25mM Tris, 200mM glycine)

M5: filter paper

S1: Prepare two pieces of filter paper and a piece of PVDF membrane.

S2: Soak filter papers in 1X transfer buffer. Place a piece of filter paper on the semi-dry transfer cell.

S3: Soak a piece of PVDF membrane in methanol for 30s. Then soak it in 1X transfer buffer for 1min. Place it on top of the filter paper.

S4: Take down polyacrylamide gel from the chamber. Place the gel on top of the PVDF membrane. Rinse them with a little 1X transfer buffer.

S5: Place another piece of filter paper on top of the gel. Carefully remove bubbles between each layer by gently squeezing them using a small rod.

S6: Set up the semi-dry transfer cell. Start transferring at 90mA and 200V (1.5mA/cm^2, constant current) for 90min.

(-) Cathode
Filter paper (upper)
Gel
PVDF membrane
Filter paper (lower)
(+) Anode

P21: Blocking

M1: PVDF membrane from P20

M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)

M3: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)

S1: Place the PVDF membrane in a container and fully soak in 1X gelatin-NET buffer. Place it on a shaker at 100rpm at room temperature for 1hr.
S2: Discard gelatin-NET buffer. Add some 1X TBST buffer to the container for washing. Place it on a shaker at 100rpm at room temperature for 15min.
S3: Discard the buffer and replace it with fresh 1X TBST buffer. Repeat the washing steps for two more times.

P22: Immunostaining

M1: PVDF membrane from P21
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: Mouse Anti-His-probe (H-3) monoclonal antibody (SANTA CRUZ #SC-8036)
M4: Mouse Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich #F3165)
M5: Mouse Anti-Myc Tag (9B11) monoclonal antibody (Cell Signaling #2276)
M6: Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Jackson ImmunoResearch #115-035-003)
M7: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)
M8: Luminol reagent (Millipore #WBKLS0500)
M9: Peroxide solution (Millipore #WBKLS0500)

S1: Slice the PVDF membrane to an appropriate size that retains the target protein area. Place the sliced membrane in a new container.
S2: Dilute primary antibody. For each antibody, dilute 5μL antibody in 10mL gelatin-NET (1:2000).
S3: Add 10mL diluted antibody solution to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at 4℃ overnight.

240814

S4: Discard the antibody solution. Wash the membrane with 1X TBST three times.

S5: Dilute secondary antibody. Dilute 3μL anti-mouse IgG antibody in 30mL gelatin-NET (1:10000).

S6: Add 10mL diluted anti-mouse IgG antibody to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at room temperature for 1hr.

S7: Discard the antibody solution. Wash the membrane with 1X TBST three times.

S8: Place the membrane on a transparent plastic board. Mix 500μL luminol reagent and 500μL peroxide solution and rinse the membrane with the mixture for 1min. The results were captured and analyzed using Biospectrum MultiSpectral Imaging System (UVP BioSpectrum 800).

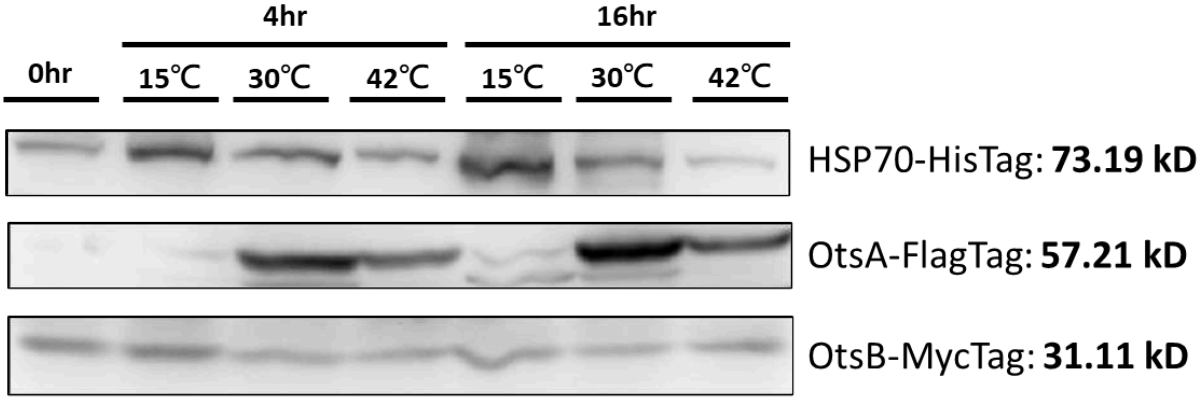
R1: Target protein molecular weight:

- HSP70-HisTag: 73.19 kDa
- TEE-OtsA-FlagTag: 57.21 kDa
- OtsB-MycTag: 31.11 kDa

R2: Since the antibodies used in western blotting were tag protein targeting antibodies, few other proteins were also stained on the membrane. Based on the molecular weight of each target protein, we assumed that the bands indicated below were target proteins.

R3: The results did not match our expectations. Chances are the protein samples were reversely prepared on 15℃ and 42℃ groups when harvesting bacteria.

R4: Repeat the experiment again.



240813

P23: CBR staining

M1: SDS-polyacrylamide gel from P19

M2: CBR solution (2.2mM Coomassie brilliant blue R-250, 45.45% methanol, 9% acetic acid)

M3: Destaining solution (20% methanol, 10% acetic acid)

M4: ddH2O

S1: Place the gel in CBR solution and shake at 100 rpm at room temperature for 10min.

S2: Discard the CBR solution. Place the gel in a destaining solution and shake for 30min for washing.

S3: Discard the destaining solution. Repeat the washing step again.

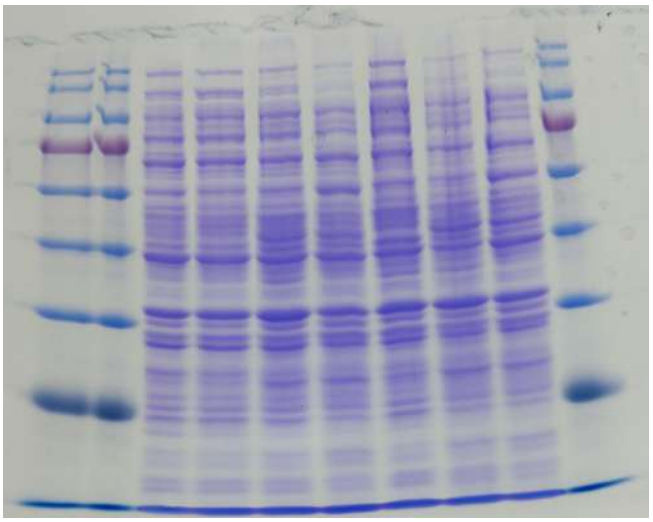
S4: Discard the destaining solution. Place the gel in ddH2O for washing overnight.

240814

S5: Place the gel on an illuminator. Take photos.

R1: CBR staining experiment was conducted to verify harvesting quantitative proteins between each group. Also, if target proteins were expressed significantly, they could be observed on the gel before conducting immunostaining experiments.

R2: CBR stained gel.



[Formosa_101] [20240815] Target Protein Expression

Purpose: To express target protein from plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" & "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" using E. coli BL21 STAR (DE3)

240815

P1: Protein expression

M1: bacterial glycerol stock from [20240726] P16 #2 (pHot+pCold)

M2: Colonies on agar plate from [20240726] P5 (pCold & pHot plates)

M3: LB medium

M4: Ampicillin stock (100mg/mL)

M5: Kanamycin stock (50mg/mL)

M6: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)

M7: 5X sample buffer (50% glycerol, 10%SDS, 250mM Tris, 0.05% bromophenol blue, 5% β-Mercaptoethanol, pH=6.8)

M8: Protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer)

S1: Inoculate 0.5mL glycerol stock (pHot+pCold) or a single colony (pHot or pCold) to 10mL LB medium containing 50μg/mL Kanamycin and 100μg/mL Ampicillin. Incubate at 30℃ overnight.

240816

S2: Measure OD600 value of each overnight culture. Dilute the overnight cultures to OD600 value~=0.1 with fresh LB medium (Kana+/Amp+). Dispense the culture to 3 flasks, each contains 20mL. Label them as “20℃”, “30℃”, and “42℃”, respectively.

S3: Harvest “0h” sample.

S4: Incubate the cultures at 20℃, 30℃, or 42℃ at 150 rpm and harvest bacteria after 4, 8, 12, and 24hr.

S5: Measure OD600 value of each sample before harvesting.

S6: Harvest bacteria with the amount equal to 1mL of OD600=1.0 culture by centrifuging the bacterial culture at 4℃ at 6000g for 5min. Harvest two tubes for each bacterial sample. Then discard the supernatant.

S7: Resuspend bacterial pellets with 1mL TBS buffer for washing. Centrifuge at 4℃ at 6000g for 5min. Discard the supernatant. Store one of the harvested bacteria of each sample at -20℃ for further fluorescent microscopy observations.

S8: Resuspend another harvested pellet of each sample with 100μL protein extraction buffer (80% TBS buffer+ 20% 5X sample buffer).

S9: Incubate each sample at 100℃ for 10min. After cooling down to room temperature, store them at -20℃ for western blotting analysis.

R1: OD600 value of overnight culture:

- pHot+pCold: 0.67
- pHot: 1.73
- pCold: 1.95

R2: OD600 value of each pHot+pCold culture after protein induction:

- 0h: 0.10
- 4h-20: 0.23
- 4h-30: 1.16
- 4h-42: 1.38
- 8h-20: 0.39
- 8h-30: 2.16
- 8h-42: 2.08
- 12h-20: 0.62
- 12h-30: 2.88
- 12h-42: 2.12
- 16h-20: 2.52
- 16h-30: 4.45
- 16h-42: 3.64

R3: OD600 value of each pHot culture after protein induction:

- 0h: 0.10
- 4h-20: 0.27
- 4h-30: 1.20
- 4h-42: 1.36
- 8h-20: 0.38
- 8h-30: 2.32
- 8h-42: 2.16
- 12h-20: 0.62
- 12h-30: 3.04
- 12h-42: 2.04
- 16h-20: 2.49
- 16h-30: 4.55
- 16h-42: 3.84

R4: OD600 value of each pCold culture after protein induction:

- 0h: 0.10
- 4h-20: 0.22
- 4h-30: 0.80
- 4h-42: 1.16
- 8h-20: 0.32
- 8h-30: 2.60
- 8h-42: 1.76
- 12h-20: 0.52
- 12h-30: 2.32
- 12h-42: 2.32
- 16h-20: 1.71
- 16h-30: 4.20
- 16h-42: 4.00

R5: To obtain quantitative bacterial protein samples, about 1.0 = 8 x 10^8 bacteria cells (~1mL of OD600=1.0 bacterial culture) were harvested with 100μL protein extraction buffer.

P2: Preparing bacterial glycerol stock

M1: bacterial culture P1 (pHot & pCold)

M2: LB medium

M3: Ampicillin stock (100mg/mL)

M4: Kanamycin stock (50mg/mL)

M5: 50% glycerol (sterilized)

S1: Prepare 10 cryogenic vials. Freeze 5 vials for both pHot and pCold overnight culture. For each vial, mix 750μL overnight culture and 750μL glycerol stock. Freeze them at -80℃.

240819

P3: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P1 (pHot+pCold samples only)

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up 2 electrophoresis chambers. One for western blotting and one for CBR staining.

S2: Prepare 15mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 5mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P1. Load 10μL of each sample and 10μL pre-stained protein markers to both gels. Sample orders were identical on both gels: marker, "CH0h", "CH4h-42", "CH8h-42", "CH4h-30", "CH8h-30", "CH12h-30", "CH24h-30", "CH8h-20", "CH12h-20", "CH24h-20", marker.

S9: Run the electrophoresis at 40mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

P4: Transferring

M1: SDS-polyacrylamide gel from P3

M2: PVDF membrane

M3: methanol

M4: 1X transfer buffer (25mM Tris, 200mM glycine)

M5: filter paper

S1: Prepare two pieces of filter paper and a piece of PVDF membrane.

S2: Soak filter papers in 1X transfer buffer. Place a piece of filter paper on the semi-dry transfer cell.

S3: Soak a piece of PVDF membrane in methanol for 30s. Then soak it in 1X transfer buffer for 1min. Place it on top of the filter paper.

S4: Take down polyacrylamide gel from the chamber. Place the gel on top of the PVDF membrane. Rinse them with a little 1X transfer buffer.
S5: Place another piece of filter paper on top of the gel. Carefully remove bubbles between each layer by gently squeezing them using a small rod.
S6: Set up the semi-dry transfer cell. Start transferring at 90mA and 200V (1.5mA/cm^2, constant current) for 90min.

(-) Cathode
Filter paper (upper)
Gel
PVDF membrane
Filter paper (lower)
(+) Anode

P5: Blocking

M1: PVDF membrane from P4
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)

S1: Place the PVDF membrane in a container and fully soak in 1X gelatin-NET buffer. Place it on a shaker at 100rpm at room temperature for 1hr.
S2: Discard gelatin-NET buffer. Add some 1X TBST buffer to the container for washing. Place it on a shaker at 100rpm at room temperature for 15min.
S3: Discard the buffer and replace it with fresh 1X TBST buffer. Repeat the washing steps for two more times.

P6: Immunostaining

M1: PVDF membrane from P5
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: Mouse Anti-His-probe (H-3) monoclonal antibody (SANTA CRUZ #SC-8036)
M4: Mouse Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich #F3165)
M5: Mouse Anti-Myc Tag (9B11) monoclonal antibody (Cell Signaling #2276)
M6: Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Jackson ImmunoResearch #115-035-003)
M7: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)
M8: Luminol reagent (Millipore #WBKLS0500)
M9: Peroxide solution (Millipore #WBKLS0500)

S1: Slice the PVDF membrane to an appropriate size that retains the target protein area. Place the sliced membrane in a new container.
S2: Dilute primary antibody. For each antibody, dilute 5μL antibody in 10mL gelatin-NET (1:2000).
S3: Add 10mL diluted antibody solution to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at 4℃ overnight.

240820

S4: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S5: Dilute secondary antibody. Dilute 3μL anti-mouse IgG antibody in 30mL gelatin-NET (1:10000).
S6: Add 10mL diluted anti-mouse IgG antibody to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at room temperature for 1hr.
S7: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S8: Place the membrane on a transparent plastic board. Mix 500μL luminol reagent and 500μL peroxide solution and rinse the membrane with the mixture for 1min. The results were

captured and analyzed using Biospectrum MultiSpectral Imaging System (UVP BioSpectrum 800).

R1: Target protein molecular weight:

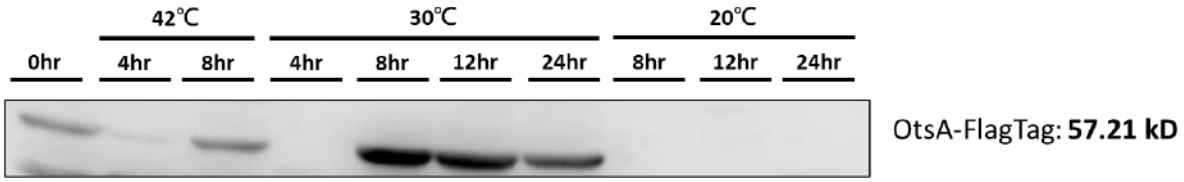
- HSP70-HisTag: 73.19 kDa
- TEE-OtsA-FlagTag: 57.21 kDa
- OtsB-MycTag: 31.11 kDa

R2: Since the antibodies used in western blotting were tag protein targeting antibodies, few other proteins were also stained on the membrane. Based on the molecular weight of each target protein, we assumed that the bands indicated below were target proteins.

R3: The results of anti-His antibody & anti-Myc antibody are not shown here due to unclear bands after capturing.

R4: The results of the OtsA expression did not match our expectations again.

R5: Repeat immunostaining on these two targets. Also, additionally perform western blotting for pHot and pCold protein samples next time.



240819

P7: CBR staining

M1: SDS-polyacrylamide gel from P3

M2: CBR solution (2.2mM Coomassie brilliant blue R-250, 45.45% methanol, 9% acetic acid)

M3: Destaining solution (20% methanol, 10% acetic acid)

M4: ddH2O

S1: Place the gel in CBR solution and shake at 100 rpm at room temperature for 10min.

S2: Discard the CBR solution. Place the gel in a destaining solution and shake for 30min for washing.

S3: Discard the destaining solution. Repeat the washing step again.

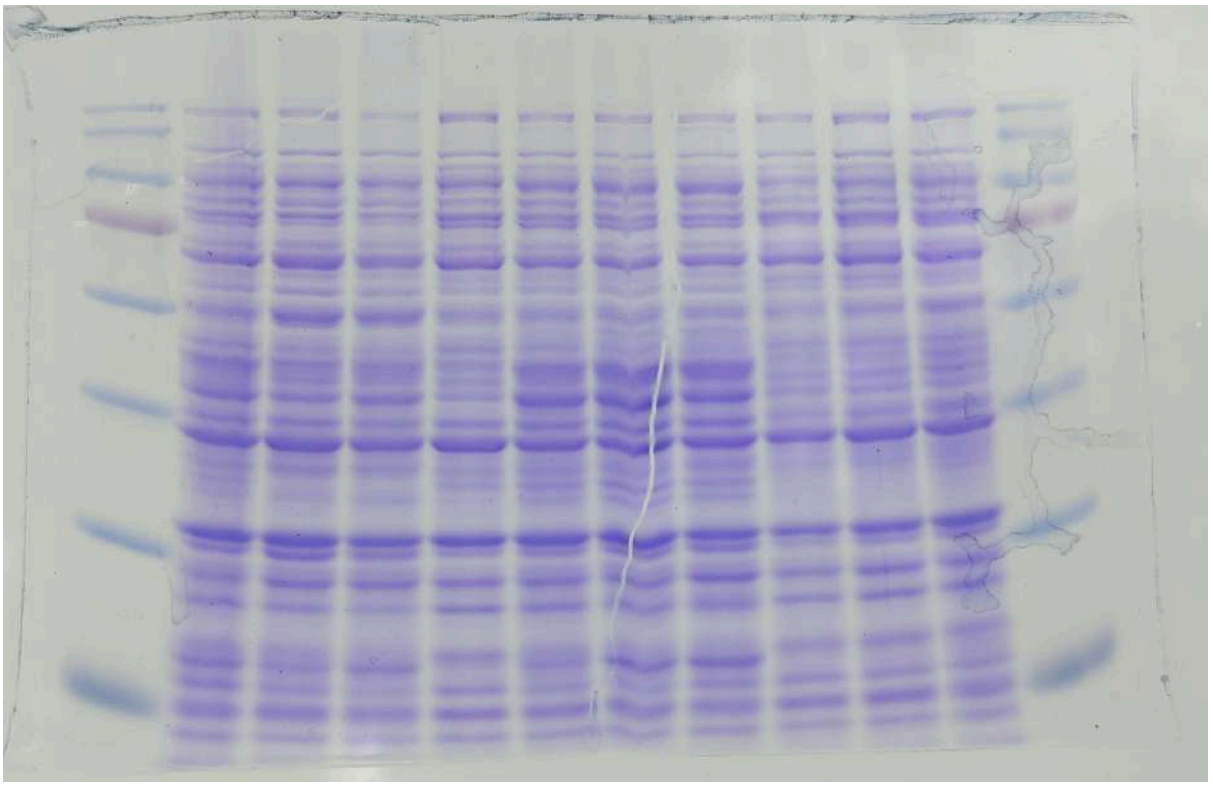
S4: Discard the destaining solution. Place the gel in ddH2O for washing overnight.

240820

S5: Place the gel on an illuminator. Take photos.

R1: CBR staining experiment was conducted to verify harvesting quantitative proteins between each group. Also, if target proteins were expressed significantly, they could be observed on the gel before conducting immunostaining experiments.

R2: CBR stained gel.



P8: Fluorescent microscopy observations

M1: Harvested bacterial pellets from P1

M2: 1X Tris buffered saline (1X TBS) buffer (20mM Tris, 150mM NaCl, pH=7.6)

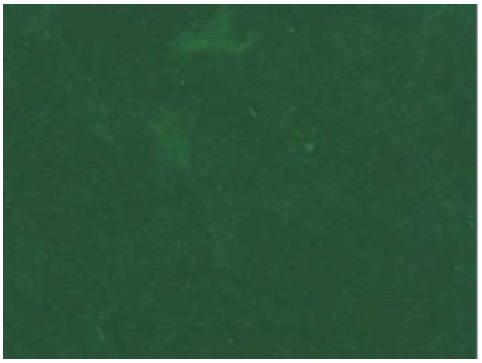
S1: Resuspend each pellet with 100μL TBS. Transfer it to a 96-well plate.

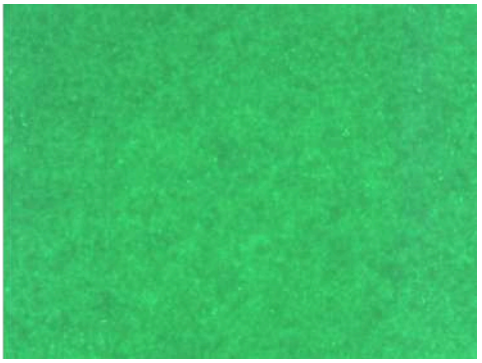
S2: Observe fluorescence using fluorescent microscope.

R1: Green fluorescence was observed in pCold groups only, which showed a trend that the lower the induction temperature was, the brighter it was.

R2: No red fluorescence was observed in any group.

R3: "pCold 0hr" vs. "pCold 24hr 20℃" under 400X magnification.





	pHot											
	4hr			8hr			12hr			24hr		
	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃
GFP	X	X	X	X	X	X	X	X	X	X	X	X
RFP	X	X	X	X	X	X	X	X	X	X	X	X

	pCold											
	4hr			8hr			12hr			24hr		
	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃
GFP	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲	▲
RFP	X	X	X	X	X	X	X	X	X	X	X	X

	pHot+pCold											
	4hr			8hr			12hr			24hr		
	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃	42℃	30℃	20℃
GFP	X	X	X	X	X	X	X	X	X	X	X	X
RFP	X	X	X	X	X	X	X	X	X	X	X	X

240822

P9: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P1 (pHot+pCold samples only)

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up 3 electrophoresis chambers.

S2: Prepare 20mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 10mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P1. Load 10μL of each sample and 10μL pre-stained protein markers to each gel. Sample orders in first gel: marker, "CH0h", "CH4h-42", "CH8h-42", "CH4h-30", "CH8h-30", "CH12h-30", "CH24h-30", "CH8h-20", "CH12h-20", "CH24h-20", marker. Sample orders in second gel: marker, "H0h", "H4h-42", "H8h-42", "H12h-42", "H24h-42", "H4h-30", "H8h-30", "H12h-30", "H24h-30", marker. Sample orders in third gel: marker, "C0h", "C4h-30", "C8h-30", "C12h-30", "C24h-30", "C4h-20", "C8h-20", "C12h-20", "C24h-20", marker.

S9: Run the electrophoresis at 60mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

P10: Transferring

M1: SDS-polyacrylamide gel from P9

M2: PVDF membrane

M3: methanol

M4: 1X transfer buffer (25mM Tris, 200mM glycine)

M5: filter paper

S1: Prepare two pieces of filter paper and a piece of PVDF membrane.

S2: Soak filter papers in 1X transfer buffer. Place a piece of filter paper on the semi-dry transfer cell.

S3: Soak a piece of PVDF membrane in methanol for 30s. Then soak it in 1X transfer buffer for 1min. Place it on top of the filter paper.
S4: Take down polyacrylamide gel from the chamber. Place the gel on top of the PVDF membrane. Rinse them with a little 1X transfer buffer.
S5: Place another piece of filter paper on top of the gel. Carefully remove bubbles between each layer by gently squeezing them using a small rod.
S6: Set up the semi-dry transfer cell. Start transferring at 270mA and 200V (1.5mA/cm^2, constant current) for 90min.

(-) Cathode
Filter paper (upper)
Gel
PVDF membrane
Filter paper (lower)
(+) Anode

P11: Blocking

M1: PVDF membrane from P10
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)

S1: Place the PVDF membrane in a container and fully soak in 1X gelatin-NET buffer. Place it on a shaker at 100rpm at room temperature for 1hr.
S2: Discard gelatin-NET buffer. Add some 1X TBST buffer to the container for washing. Place it on a shaker at 100rpm at room temperature for 15min.

S3: Discard the buffer and replace it with fresh 1X TBST buffer. Repeat the washing steps for two more times.

P12: Immunostaining

M1: PVDF membrane from P11
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: Mouse Anti-His-probe (H-3) monoclonal antibody (SANTA CRUZ #SC-8036)
M4: Mouse Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich #F3165)
M5: Mouse Anti-Myc Tag (9B11) monoclonal antibody (Cell Signaling #2276)
M6: Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Jackson ImmunoResearch #115-035-003)
M7: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)
M8: Luminol reagent (Millipore #WBKLS0500)
M9: Peroxide solution (Millipore #WBKLS0500)

S1: Slice the PVDF membrane to an appropriate size that retains the target protein area. For gel 1, slice the area where HSP70-His & OtsB-Myc were located. For gel 2, slice the area where OtsA-Flag & OtsB-Myc were located. For gel 3, slice the area where HSP70-His was located. Place the sliced membranes in a new container.
S2: Dilute primary antibody. For each antibody, dilute 10μL antibody in 20mL gelatin-NET (1:2000).
S3: Add 10mL diluted antibody solution to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at 4℃ overnight.

240823

S4: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S5: Dilute secondary antibody. Dilute 5μL anti-mouse IgG antibody in 50mL gelatin-NET (1:10000).

S6: Add 10mL diluted anti-mouse IgG antibody to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at room temperature for 1hr.

S7: Discard the antibody solution. Wash the membrane with 1X TBST three times.

S8: Place the membrane on a transparent plastic board. Mix 500μL luminol reagent and 500μL peroxide solution and rinse the membrane with the mixture for 1min. The results were captured and analyzed using Biospectrum MultiSpectral Imaging System (UVP BioSpectrum 800).

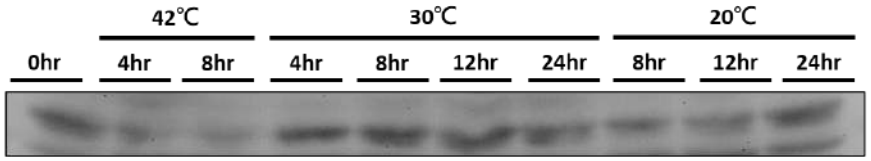
R1: Target protein molecular weight:

- HSP70-HisTag: 73.19 kDa
- TEE-OtsA-FlagTag: 57.21 kDa
- OtsB-MycTag: 31.11 kDa

R2: Since the antibodies used in western blotting were tag protein targeting antibodies, few other proteins were also stained on the membrane. Based on the molecular weight of each target protein, we assumed that the bands indicated below were target proteins.

R3: The results of anti-His antibody (Both gel 1 & gel 2) are still not shown here due to unclear bands after capturing again.

R4: The results of the OtsB expression in gel 1 did not match our expectations again. Similar to previous results in P6.



R5: The results of gel 2 are not shown here.

R6: The bands were unclear on gel 3. It is probably because of inappropriate transferring.

R7: Since we take overnight culture directly as 0hr time point sample, the expression of proteins were relatively higher than previous experiments. Without dilution and a short time of incubation, it could not stand as a control sample. For future experiments, be sure to incubate for a few hours before starting induction.

R8: All the results did not fit our expectations, indicating that target protein induction in this experiment had failed. Repeat the induction experiment again.

[Formosa_101] [20240828] Target Protein Expression

Purpose: To express target protein from plasmid "pET-9a-PibpA-HSP70-His-T2A-RFP" & "pET-3a-PcspA-otsA-Flag-T2A-otsB-Myc-T2A-EGFP" using E. coli BL21 STAR (DE3)

240828

P1: Protein expression

M1: bacterial glycerol stock from [20240726] P16 #2 (pHot+pCold)

M2: bacterial glycerol stock from [20240815] P2 (pHot, pCold)

M3: LB medium

M4: Ampicillin stock (100mg/mL)

M5: Kanamycin stock (50mg/mL)

M6: 1X phosphate buffered saline (1X PBS) buffer (137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4 ,pH=7.4)

M7: 5X sample buffer (50% glycerol, 10%SDS, 250mM Tris, 0.05% bromophenol blue, 5% β-Mercaptoethanol, pH=6.8)

M8: Protein extraction buffer (80% PBS buffer + 20% 5X sample buffer)

S1: Inoculate 1.5mL glycerol stock (pHot, pCold, and pHot+pCold) to 50mL LB medium containing 50μg/mL Kanamycin and/or 100μg/mL Ampicillin. Incubate at 30℃ for 6hr.

S2: Measure OD600 value of each culture. Dilute each culture to OD600 value = 0.4 with fresh LB medium containing corresponding antibiotics. Dispense the culture to 3 tubes, each contains 10mL. Label them as “16h-20℃”, “12h-30℃”, and “2h-42℃”, respectively.

S3: Harvest “0h” samples.

S4: Incubate the cultures at 42℃, 30℃, or 20℃ at 150 rpm for 2hr, 12hr, and 16hr, respectively.

240829

S5: Measure OD600 value of each sample before harvesting.

S6: Harvest bacteria with the amount equal to 1mL of OD600=1.0 culture by centrifuging the bacterial culture at 4℃ at 6000g for 5min. Harvest two tubes for each bacterial sample. Then discard the supernatant.

S7: Resuspend bacterial pellets with 1mL PBS buffer for washing. Centrifuge at 4℃ at 6000g for 5min. Discard the supernatant. Store one of the harvested bacteria of each sample at -20℃ for further fluorescent microscopy observations.

S8: Resuspend another harvested pellet of each sample with 100μL protein extraction buffer (80% PBS buffer+ 20% 5X sample buffer).

S9: Incubate each sample at 100℃ for 10min. After cooling down to room temperature, store them at -20℃ for western blotting analysis.

R1: OD600 value of each pHot+pCold culture after protein induction:

- 0h: 0.40
- 2h-42: 1.04
- 12h-30: 1.24
- 16h-20: 0.90

R2: OD600 value of each pHot culture after protein induction:

- 0h: 0.40
- 2h-42: 1.32
- 12h-30: 1.44
- 16h-20: 0.16

R3: OD600 value of each pCold culture after protein induction:

- 0h: 0.40
- 2h-42: 1.50
- 12h-30: 1.46
- 16h-20: 0.04

R4: To obtain quantitative bacterial protein samples, about 1.0 = 8 x 10^8 bacteria cells (~1mL of OD600=1.0 bacterial culture) were harvested with 100μL protein extraction buffer.

240907

P2: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P1 (pHot+pCold samples only)

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up an electrophoresis chamber.

S2: Prepare 10mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 5mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P1. Load 10µL of each sample and 10µL pre-stained protein markers to the gel. Sample orders are: marker, "CH0h", "CH2h-42", "CH16h-20", "H0h", "H2h-42", "H16h-20", "C0h", "C2h-42", "C16h-20", marker.

S9: Run the electrophoresis at 20mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

P4: Transferring

M1: SDS-polyacrylamide gel from P3
M2: PVDF membrane
M3: methanol
M4: 1X transfer buffer (25mM Tris, 200mM glycine)
M5: filter paper

S1: Prepare two pieces of filter paper and a piece of PVDF membrane.
S2: Soak filter papers in 1X transfer buffer. Place a piece of filter paper on the semi-dry transfer cell.
S3: Soak a piece of PVDF membrane in methanol for 30s. Then soak it in 1X transfer buffer for 1min. Place it on top of the filter paper.
S4: Take down polyacrylamide gel from the chamber. Place the gel on top of the PVDF membrane. Rinse them with a little 1X transfer buffer.
S5: Place another piece of filter paper on top of the gel. Carefully remove bubbles between each layer by gently squeezing them using a small rod.
S6: Set up the semi-dry transfer cell. Start transferring at 90mA and 200V (1.5mA/cm^2, constant current) for 90min.

(-) Cathode
Filter paper (upper)
Gel
PVDF membrane
Filter paper (lower)

(+) Anode

P5: Blocking

M1: PVDF membrane from P4
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)

S1: Place the PVDF membrane in a container and fully soak in 1X gelatin-NET buffer. Place it on a shaker at 100rpm at room temperature for 1hr.
S2: Discard gelatin-NET buffer. Add some 1X TBST buffer to the container for washing. Place it on a shaker at 100rpm at room temperature for 15min.
S3: Discard the buffer and replace it with fresh 1X TBST buffer. Repeat the washing steps for two more times.

P6: Immunostaining

M1: PVDF membrane from P5
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: Mouse Anti-His-probe (H-3) monoclonal antibody (SANTA CRUZ #SC-8036)
M4: Mouse Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich #F3165)
M5: Mouse Anti-Myc Tag (9B11) monoclonal antibody (Cell Signaling #2276)
M6: Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Jackson ImmunoResearch #115-035-003)
M7: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)
M8: Luminol reagent (Millipore #WBKLS0500)
M9: Peroxide solution (Millipore #WBKLS0500)

S1: Slice the PVDF membrane to an appropriate size that retains the target protein area. Place the sliced membrane in a new container.
S2: Dilute primary antibody. For each antibody, dilute 5μL antibody in 10mL gelatin-NET (1:2000).
S3: Add 10mL diluted antibody solution to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at 4℃ overnight.

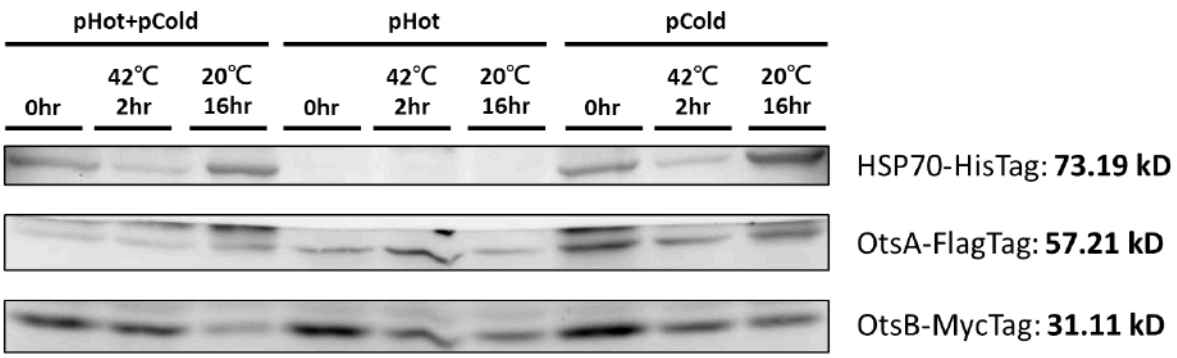
240908

S4: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S5: Dilute secondary antibody. Dilute 3μL anti-mouse IgG antibody in 30mL gelatin-NET (1:10000).
S6: Add 10mL diluted anti-mouse IgG antibody to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at room temperature for 1hr.
S7: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S8: Place the membrane on a transparent plastic board. Mix 500μL luminol reagent and 500μL peroxide solution and rinse the membrane with the mixture for 1min. The results were captured and analyzed using Biospectrum MultiSpectral Imaging System (UVP BioSpectrum 800).

R1: Target protein molecular weight:

- HSP70-HisTag: 73.19 kDa
- TEE-OtsA-FlagTag: 57.21 kDa
- OtsB-MycTag: 31.11 kDa

R2: Since the antibodies used in western blotting were tag protein targeting antibodies, few other proteins were also stained on the membrane. Based on the molecular weight of each target protein, we assumed that the bands indicated below were target proteins.
R3: The target bands located in the predicted area do not fit our expectations, indicating that whether target protein induction failed again, or the size of target proteins might be different.
R4: If the size of these proteins were different from our predictions, then we should repeat western blotting but retain the whole PVDF membrane for immunostaining without any slicing.



240912

P7: Fluorescent microscopy observations

M1: Harvested bacterial pellets from P1

M2: 1X phosphate buffered saline (1X PBS) buffer (137mM NaCl, 2.7mM KCl, 10mM Na2HPO4, 1.8mM KH2PO4 ,pH=7.4)

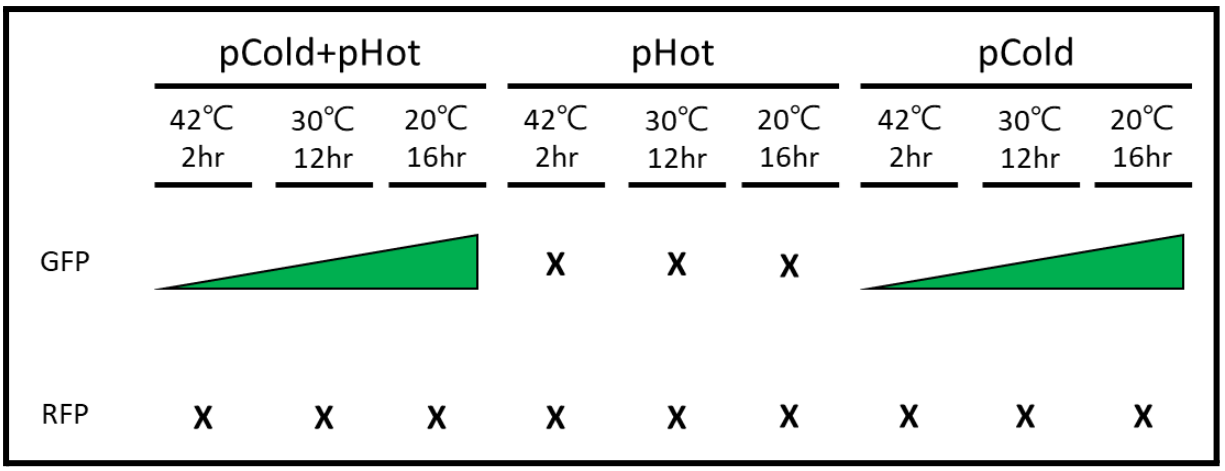
S1: Resuspend each pellet with 100μL PBS. Transfer it to a 96-well plate.

S2: Observe fluorescence using fluorescent microscope.

R1: Green fluorescence was observed in pCold+pHot and pCold groups, which showed a trend that the lower the induction temperature was, the brighter it was. These results fit our expectations.

R2: Still no red fluorescence was observed in any group.

R3: “pHot+pCold 20℃ 16hr” vs. “pHot 20℃ 16hr” vs. “pCold 20℃ 16hr” under 400X magnification.



240921

P8: SDS-PAGE

M1: Solution A (Acrylamide/Bis-Acrylamide (29:1) 30% solution)

M2: Solution B (1.5M Tris, 0.36% TEMED, pH=6.8)

M3: Solution C (0.5M Tris, 0.4% TEMED, pH-6.8)

M4: 10% sodium dodecyl sulfate (SDS)

M5: 5% ammonium persulfate (APS)

M6: ddH2O

M7: isopropanol

M8: Pre-stained protein marker (Omics Bio)

M9: Protein samples from P1 (pHot+pCold samples only)

M10: 1X SDS running buffer (25mM Tris, 190mM glycine, 1% SDS)

S1: Set up 3 electrophoresis chambers.

S2: Prepare 20mL 10% separation gel solution as described. Pour 6mL separation gel solution to each chamber.

S3: Add 1mL isopropanol to each chamber. By layering on top of the gel solution, it creates a smooth, flat surface as the gel polymerizes. Also it helps prevent formation of bubbles.

S4: After separation gel polymerization, discard isopropanol from the chamber.

S5: Prepare 10mL 4% stacking gel solution as described. Pour 2mL stacking gel solution to each chamber.

S6: Insert the well comb to the top of each chamber.

S7: After stacking gel polymerization, place two chambers into the electrophoresis apparatus. Fill the apparatus with 1X SDS running buffer. Remove the well comb.

S8: Thaw protein samples from P1. Load 10µL of each sample and 10µL pre-stained protein markers to both gels. Sample orders were identical on every gels: marker, "CH0h", "CH2h-42", "CH16h-20", "H0h", "H2h-42", "H16h-20", "C0h", "C2h-42", "C16h-20", marker.

S9: Run the electrophoresis at 60mA (constant current) for 90min.

Materials (mL)	Separation Gel						Stacking Gel
Percentage	**5%**	**7.5%**	**10%**	**12.5%**	**15%**	**20%**	**4%**
Solution A	1.65	2.5	3.35	4.15	5	6.5	0.66
Solution B	2.5	2.5	2.5	2.5	2.5	2.5	-
Solution C	-	-	-	-	-	-	1.24
10% SDS	0.1	0.1	0.1	0.1	0.1	0.1	0.05
dH2O	5.7	4.85	4	3.2	2.35	0.85	2.95
APS	0.05	0.05	0.05	0.05	0.05	0.05	0.1
Total Volume	**10**	**10**	**10**	**10**	**10**	**10**	**5**

P9: Transferring

M1: SDS-polyacrylamide gel from P8

M2: PVDF membrane

M3: methanol

M4: 1X transfer buffer (25mM Tris, 200mM glycine)

M5: filter paper

S1: Prepare two pieces of filter paper and a piece of PVDF membrane.

S2: Soak filter papers in 1X transfer buffer. Place a piece of filter paper on the semi-dry transfer cell.

S3: Soak a piece of PVDF membrane in methanol for 30s. Then soak it in 1X transfer buffer for 1min. Place it on top of the filter paper.

S4: Take down polyacrylamide gel from the chamber. Place the gel on top of the PVDF membrane. Rinse them with a little 1X transfer buffer.
S5: Place another piece of filter paper on top of the gel. Carefully remove bubbles between each layer by gently squeezing them using a small rod.
S6: Set up the semi-dry transfer cell. Start transferring at 270mA and 200V (1.5mA/cm^2, constant current) for 90min.

(-) Cathode
Filter paper (upper)
Gel
PVDF membrane
Filter paper (lower)
(+) Anode

P10: Blocking

M1: PVDF membrane from P9
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)

S1: Place the PVDF membrane in a container and fully soak in 1X gelatin-NET buffer. Place it on a shaker at 100rpm at room temperature for 1hr.
S2: Discard gelatin-NET buffer. Add some 1X TBST buffer to the container for washing. Place it on a shaker at 100rpm at room temperature for 15min.
S3: Discard the buffer and replace it with fresh 1X TBST buffer. Repeat the washing steps for two more times.

P11: Immunostaining

M1: PVDF membrane from P10
M2: Gelatin-NET buffer (50mM Tris, 2.5mg/L gelatin, 150mM NaCl, 5mM EDTA, 0.05% tween-20)
M3: Mouse Anti-His-probe (H-3) monoclonal antibody (SANTA CRUZ #SC-8036)
M4: Mouse Anti-FLAG M2 monoclonal antibody (Sigma-Aldrich #F3165)
M5: Mouse Anti-Myc Tag (9B11) monoclonal antibody (Cell Signaling #2276)
M6: Peroxidase AffiniPure™ Goat Anti-Mouse IgG (Jackson ImmunoResearch #115-035-003)
M7: 1X TBST buffer (20mM Tris, 150mM NaCl, 0.1% tween-20)
M8: Luminol reagent (Millipore #WBKLS0500)
M9: Peroxide solution (Millipore #WBKLS0500)

S1: Dilute primary antibody. For each antibody, dilute 10μL antibody in 20mL gelatin-NET (1:2000).
S2: Add 20mL diluted antibody solution to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at 4℃ overnight.

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S3: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S4: Dilute secondary antibody. Dilute 6μL anti-mouse IgG antibody in 60mL gelatin-NET (1:10000).
S5: Add 20mL diluted anti-mouse IgG antibody to the container with corresponding sliced membrane. Place it on a shaker at 100rpm at room temperature for 1hr.
S6: Discard the antibody solution. Wash the membrane with 1X TBST three times.
S7 Place the membrane on a transparent plastic board. Mix 1mL luminol reagent and 1mL peroxide solution and rinse the membrane with the mixture for 1min. The results were captured and analyzed using Biospectrum MultiSpectral Imaging System (UVP BioSpectrum 800).

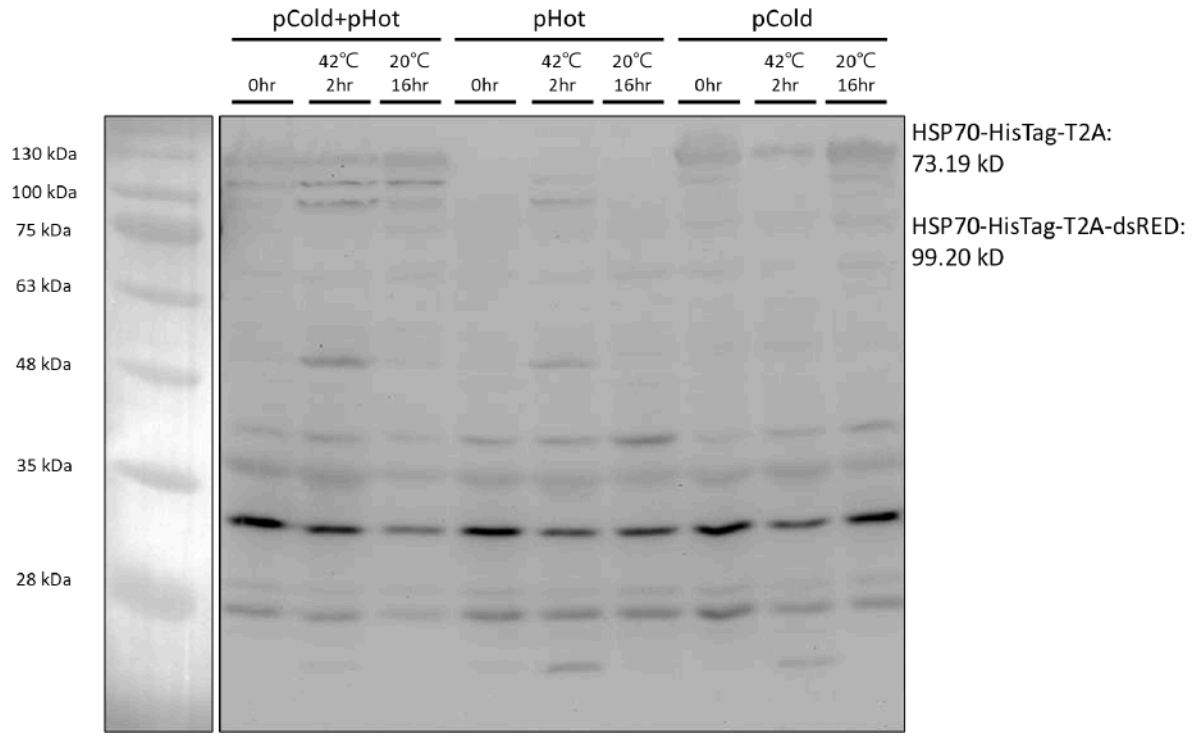
R1: Target protein molecular weight:

- HSP70-HisTag: 73.19 kDa
- TEE-OtsA-FlagTag: 57.21 kDa
- OtsB-MycTag: 31.11 kDa

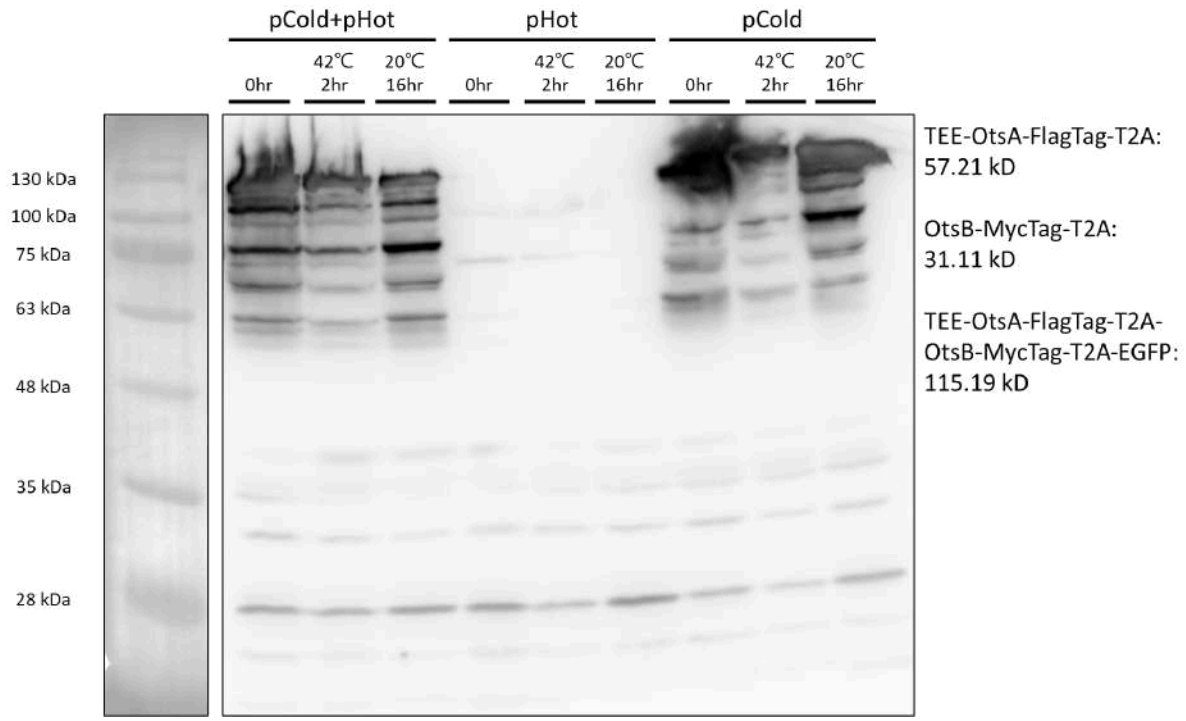
R2: Since the antibodies used in western blotting were tag protein targeting antibodies, few other proteins were also stained on the membrane. Based on the molecular weight of each target protein, we assumed that the bands indicated below were target proteins.

R3: Based on the results, we could assume that the **T2A self-cleavage sequence used for separating proteins performed poor cleavage efficiency**. Thus producing extended length of peptides. Also, the target proteins might be truncated due to unknown reasons. These circumstances caused multiple bands observed on the membrane.

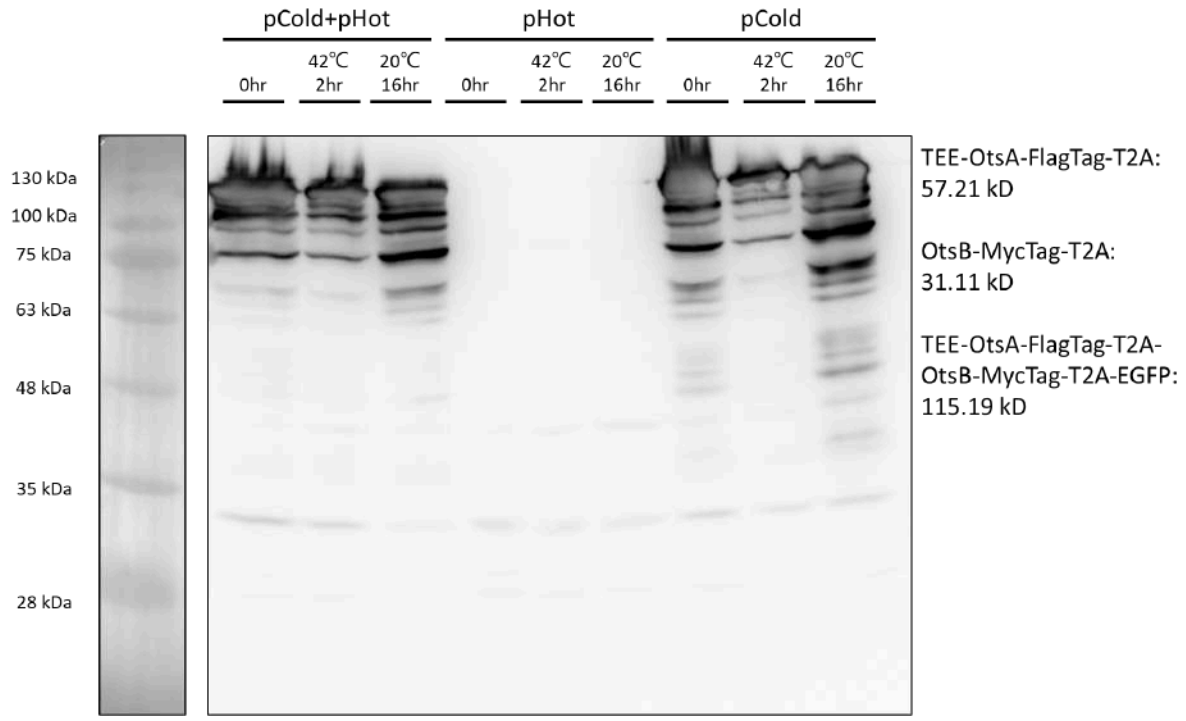
R4: The results of staining with anti-HisTag antibody.



R5: The results of staining with anti-FlagTag antibody.



R6: The results of staining with anti-MycTag antibody.



Final Results: In our design, we utilize T2A self-cleavage sequence to separate different target genes expressed by the same promoter. However, T2A sequence might not be an appropriate tool to be used in the E. coli expression system. To overcome this issue in

future, we could just simply introduce these genes with separated RBS and terminators. It may improve proper protein expressions.