Development of an equine IFNγ release assay (IGRA) for glanders

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岡山理科大学

OKAYAMA UNIVERSITY OF SCIENCE

1. The eqIFNγ DNA was synthesized by referring to the nucleotide sequence from NCBI

```
CATATGCACC ATCATCATCA TCATAGCGAT AAAATTATTC ACCTGACTGA CGACAGTTTT GACACGGATG
TACTCAAAGC GGACGGGGCG ATCCTCGTCG ATTTCTGGGC AGAGTGGTGC GGTCCGTGCA AAATGATCGC
CCCGATTCTG GATGAAATCG CTGACGAATA TCAGGGCAAA CTGACCGTTG CAAAACTGAA CATCGATCAA
AACCCTGGCA CTGCGCCGAA ATATGGCATC CGTGGTATCC CGACTCTGCT GCTGTTCAAA AACGGTGAAG
TGGCGGCAAC CAAAGTGGGT GCACTGTCTA AAGGTCAGTT GAAAGAGTTC CTCGACGCTA ACCTGGCCTA
TTATTGCCAA GCGGCGTTTT TCAAGGAGAT TGAGAACCTG AAGGAGTACT TTAACGCGAG CAACCCGGAC
GTGGGCGACG GCGGCCCGCT GTTCCTGGAT ATCCTGAAAA ACTGGAAGGA AGACAGCGAT AAAAAGATTA
TCCAGAGCCA AATCGTGAGC TTCTACTTTA AGCTGTTCGA GAACCTGAAG GACAACCAAG TGATCCAGAA
GAGCATGGAC ACCATCAAGG AAGACCTGTT CGTTAAGTTT TTCAACAGCA GCACCAGCAA ACTGGAGGAC
TTCCAGAAGC TGATTCAGAT CCCGGTTAAC GATCTGAAGG TGCAGCGTAA AGCGATCAGC GAACTGATCA
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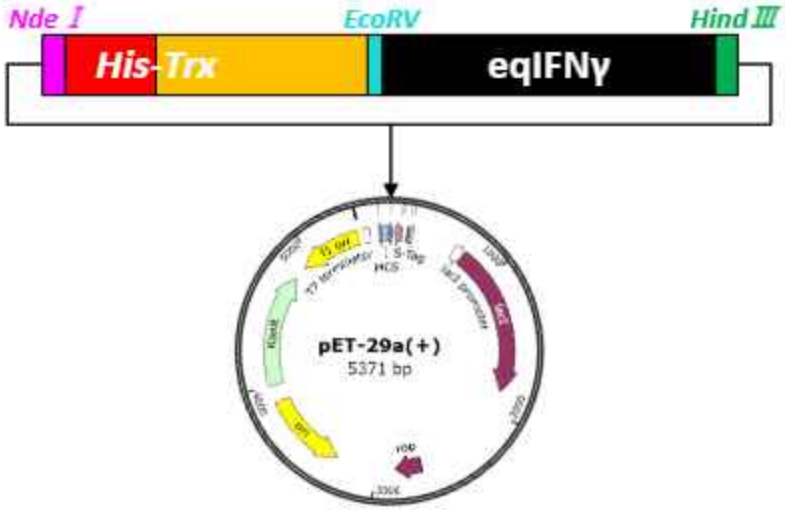
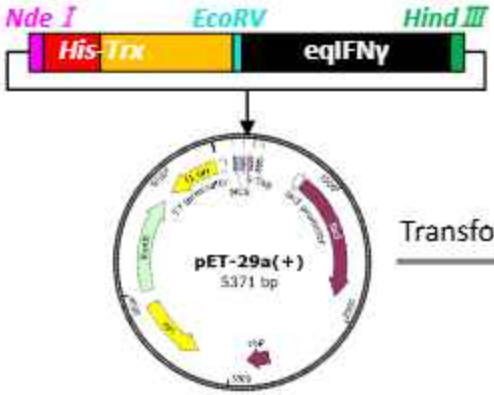
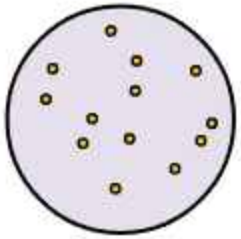


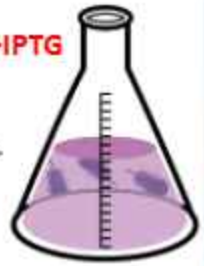
2. His-Trx_eqIFNγ and His-Trx were Inserted into the pET29a+ plasmid and the proteins were expressed, respectively

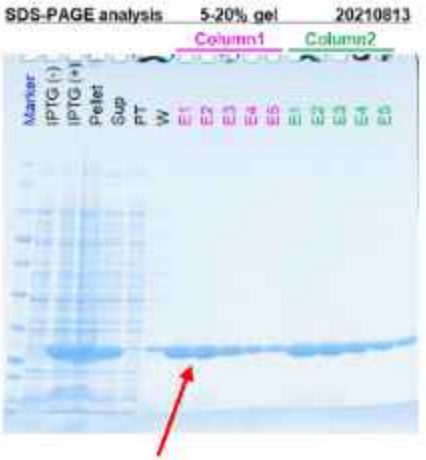


Transformation



Protein expression

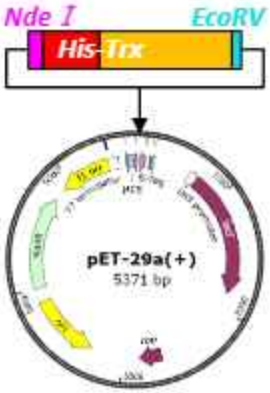




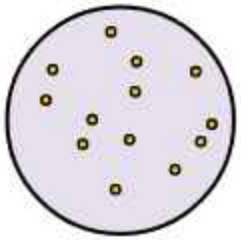
His-Trx-eqIFNγ_pET29a+

Rosetta-gami 2 (DE3) pLysS

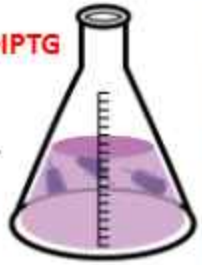
His-Trx_eqIFNγ (29.7kD)

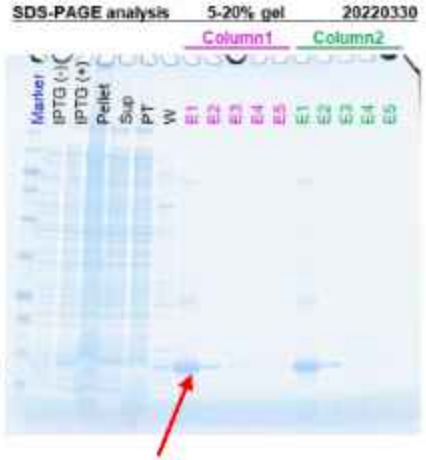


Transformation



Protein expression



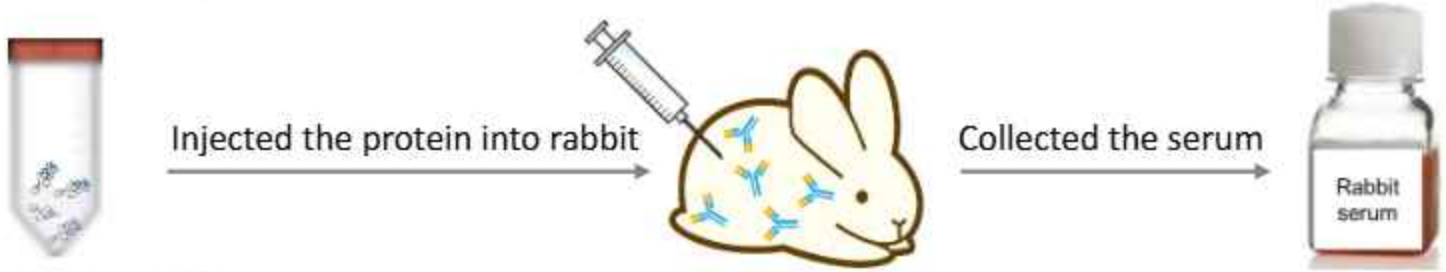


His-Trx_pET29a+

Rosetta-gami 2 (DE3) pLysS

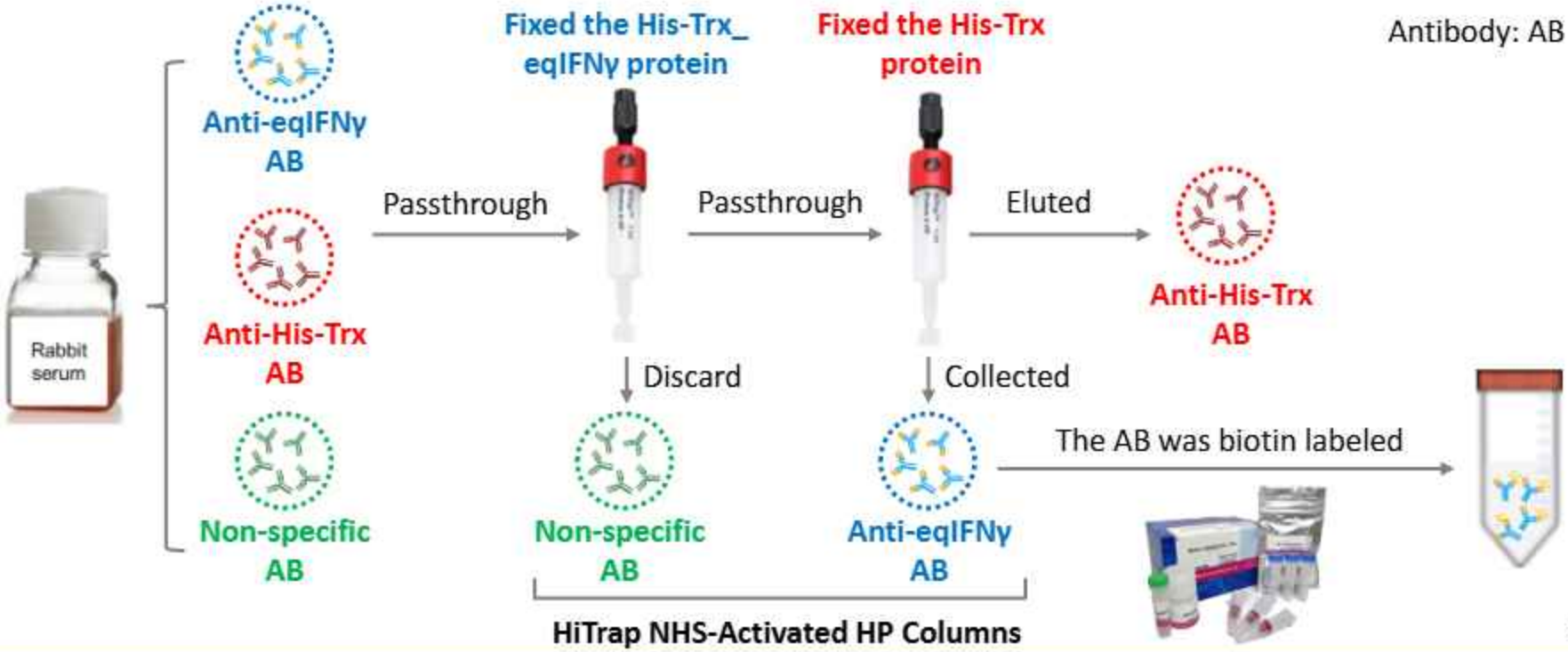
His-Trx (12.8kD)

3. Injected the protein into rabbit and collected the serum

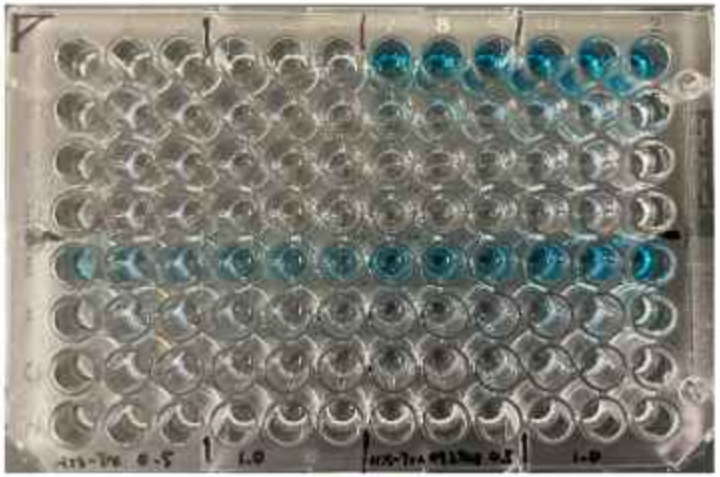


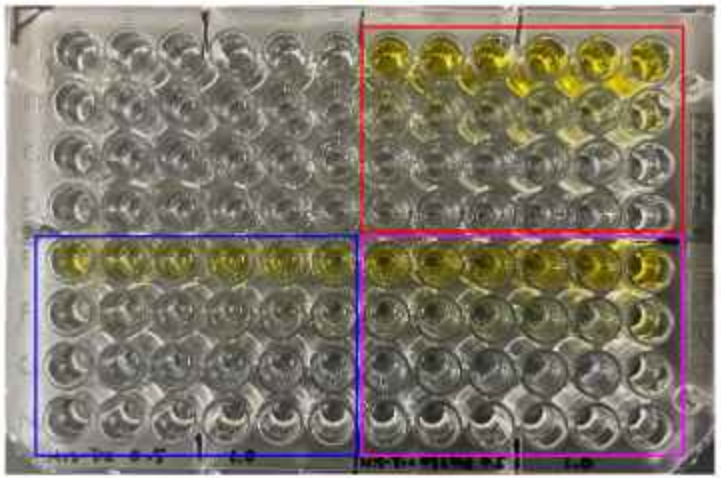
His-Trx eqIFNγ protein

4. Specific AB were purified from serum, and the anti-eqIFNγ AB was biotin labeled

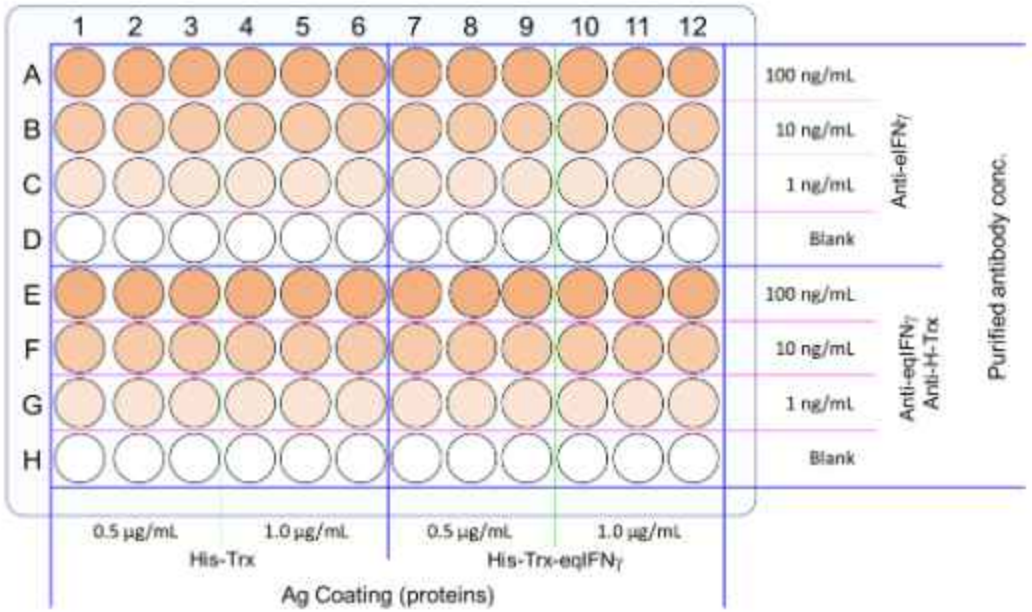


5. The eqIFNγ protein specific antibody was confirmed by ELISA method





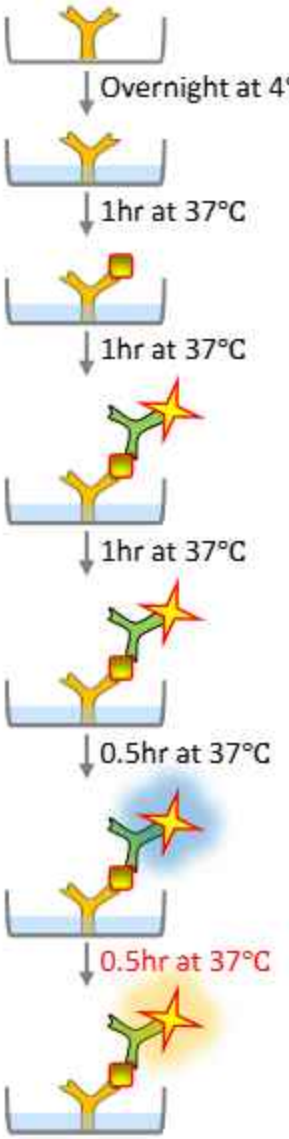
The binding reaction between His-Trx protein and His-Trx-specific antibody



The binding reaction between His-Trx-eqIFNγ protein and eqIFNγ-specific antibody

The binding reaction between His-Trx-eqIFNγ protein and His-Trx-specific antibody

6. ELISA Development of IGRA for glanders



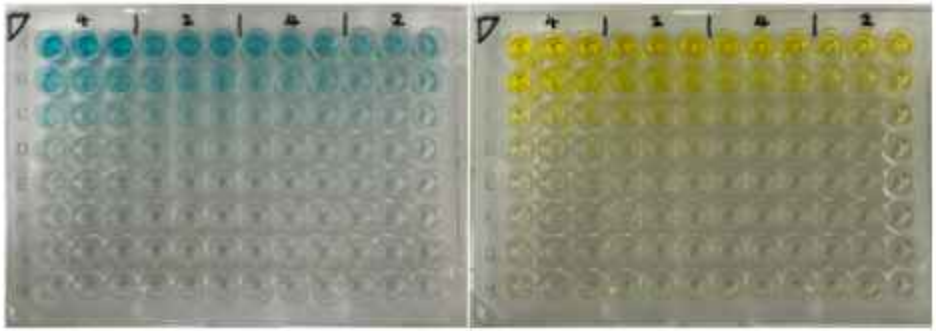
- Capture Ab: Anti-eqIFNγ Ab
- Blocking: Block Ace
- Wash, add standard
  His-Trx eqIFNγ protein
- Wash, add detection Ab
  Biotin labeled anti-eqIFNγ Ab
- Wash, add Ultra-Sensitive
  ABC Peroxidase
- Wash, add TMB
  Substrate Solution
- Stopped the reaction
  Read the OD value (450nm)

Table1. Coating the Biotin non-labeled anti-eqIFNγ AB into the 96 well plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	Coating anti-eqIFNγ AB 4ug/ml			Coating anti-eqIFNγ AB 2ug/ml			Coating anti-eqIFNγ AB 4ug/ml			Coating anti-eqIFNγ AB 2ug/ml		
B												
C												
D												
E												
F												
G												
H												

Table2. Dilution of standard (His-Trx eqIFNγ protein)

Standard	St1	St2	St3	St4	St5	St6	St7	Blank
Conc. (ng/ml)	30	10	3	1	0.3	0.1	0.03	0



Reaction was slower than in general ELISA

1. To improve sensitivity and specificity, eqIFNγ was expressed as three fragments

eqIFNγ1

QAAFFKE IENLKEYFNA SNPDVGDGGP LFLDILKNWK EDSDKKIIQS

eqIFNγ2

QIVSFYFKLF ENLKDNQVIQ KSMDTIKEDL FVKFFNSSTS KLEDFQKLIQ

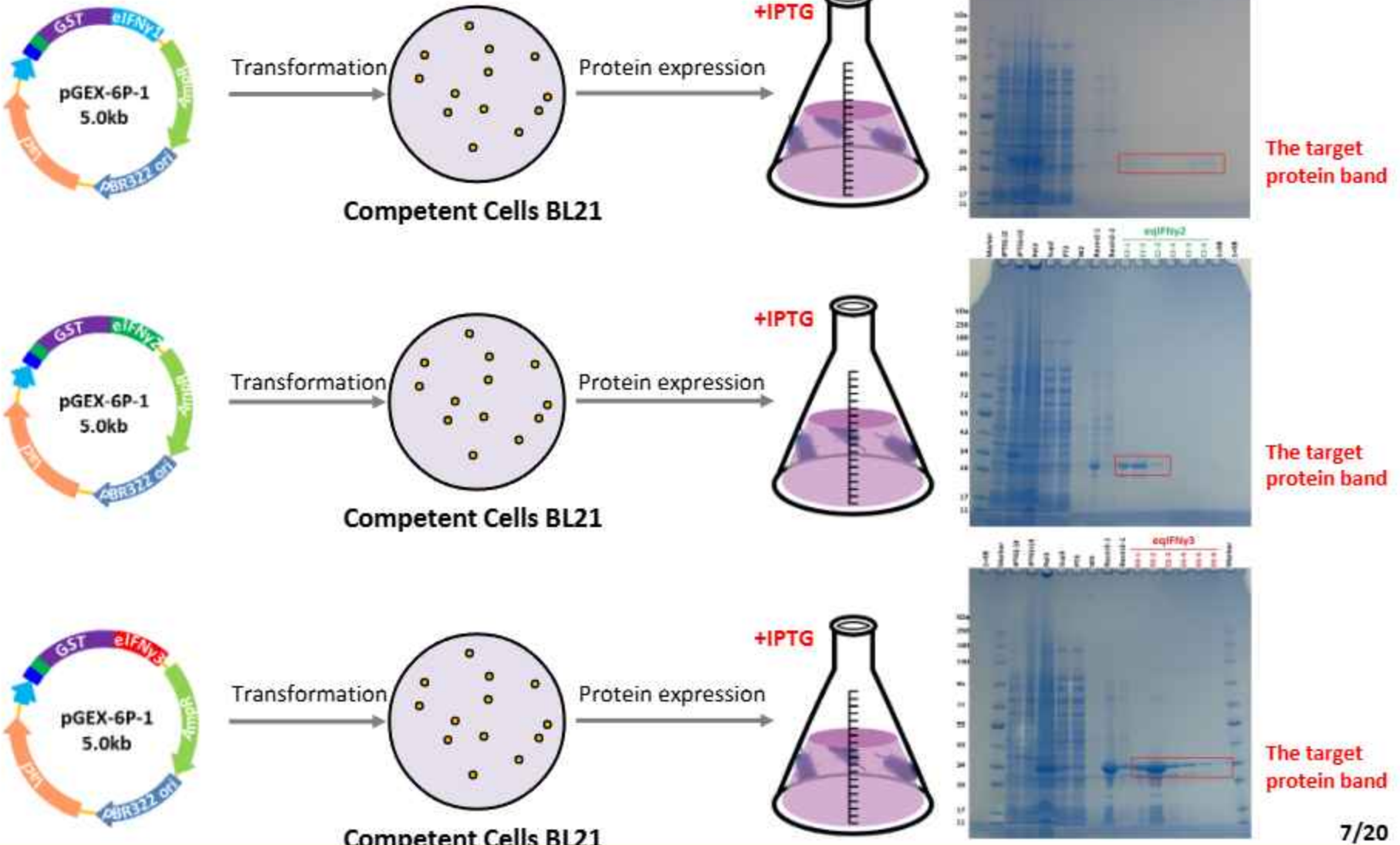
eqIFNγ3

IPVNDLKVQR KAISELIKVM NDLSPKANLR KRKRSQNPFR GRRALQ

1) Fragmented the eqIFNγ cDNA into three parts: eqIFNγ1, eqIFNγ2, and eqIFNγ3.
2) Inserted them into pGEX-6p-1 plasmid for individual protein expression.

⇒ To separately purify specific antibodies against epitopes on each fragment protein and develop a high-sensitivity, high-specificity ELISA assay.

1-1. Purification of eqIFNγ2 and 3 was successful, but eqIFNγ1 was not purified



1-2. Purified eqIFNγ1 under denaturing conditions and then performed dialysis

1) The cells were lysed in 6 M Guanidine hydrochloride, 50 mM Tris-HCl (pH 8.2), and the protein purified.

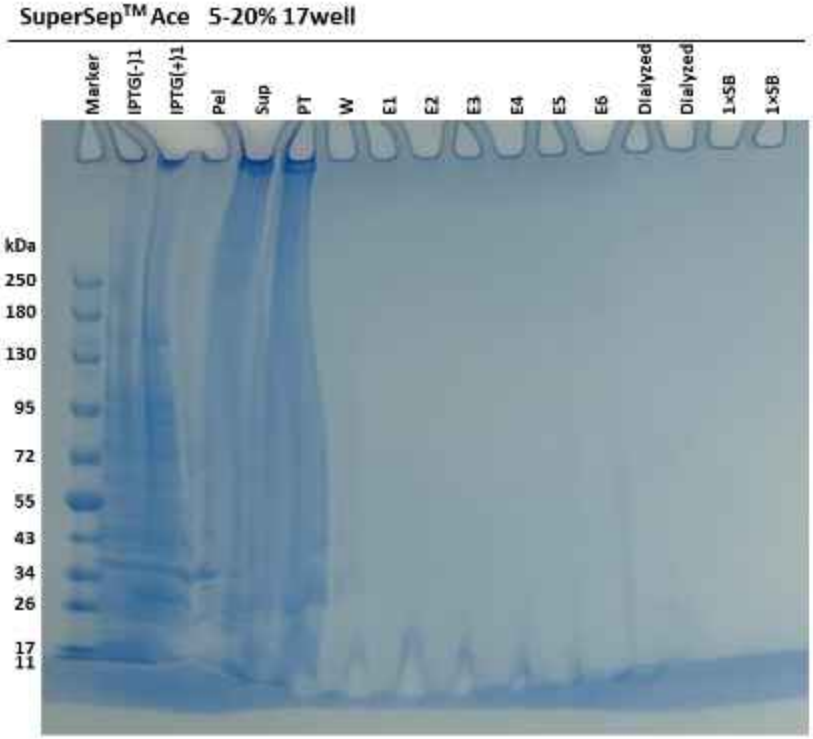
2) Supernatant after sonication was dialyzed in three steps.

(1) Dialyzed overnight in 8 M urea - 50 mM Tris-HCl (pH 8.0).

(2) Dialyzed overnight in 1 M urea - 50 mM Tris-HCl (pH 8.0).

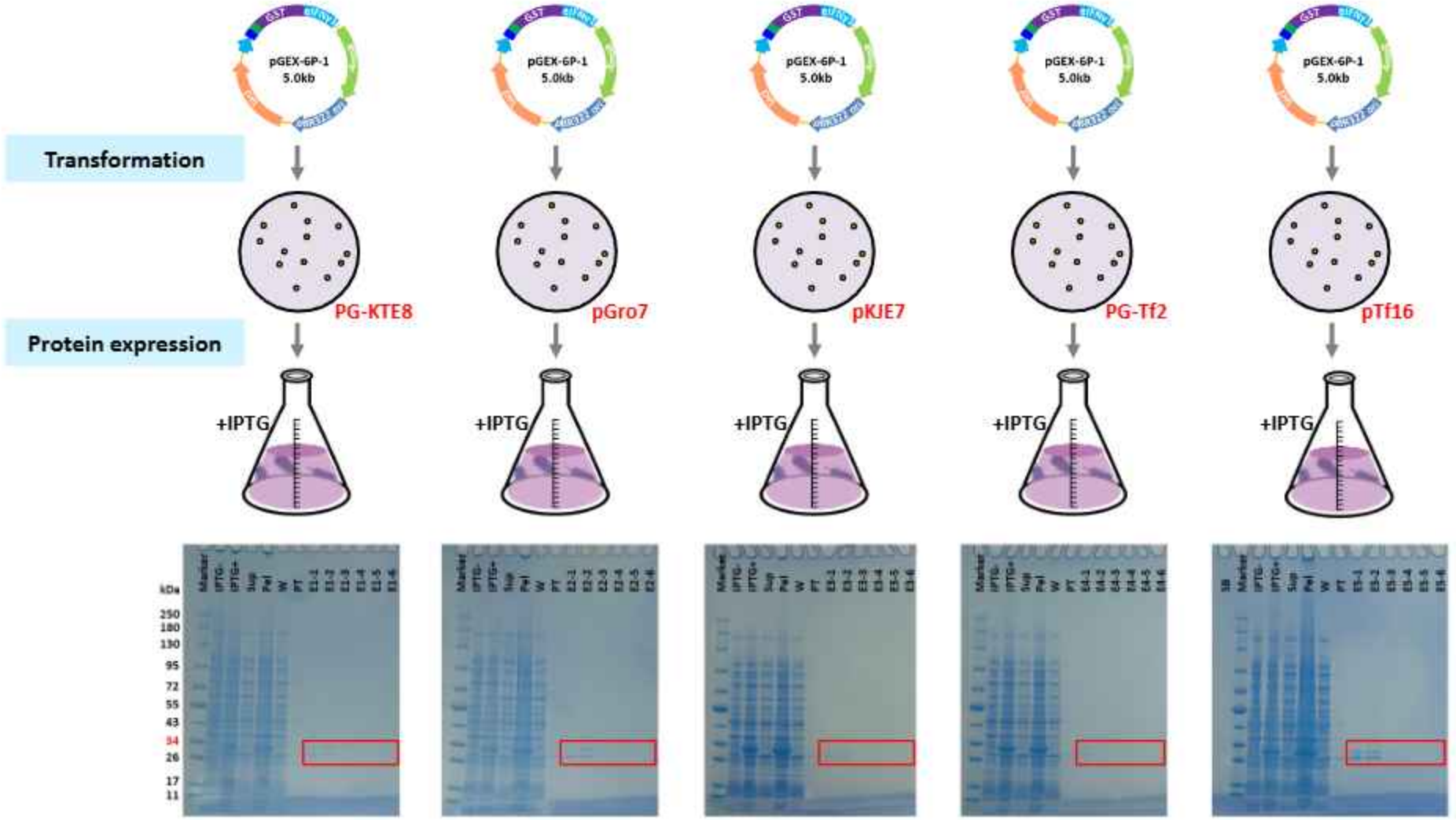
(3) Dialyzed overnight in 50 mM urea - 50 mM Tris-HCl (pH 8.0).

Recovered the protein.



The target band was not detected in the SDS-PAGE analysis.

1-3. eqIFNγ1 was expressed & purified using Chaperone Competent Cells BL21 Set



Co-expression with five chaperone plasmids was performed to promote solubilization, but it was unsuccessful.

2. eqIFNγ was expressed in mammalian cells

HindIII-eqIFNg-HAT-EcoRI

HAT (Lys-Asp-His-Leu-Ile-His-Asn-Val-His-Lys-Glu-Glu-His-Ala-His-Ala-His-Asn-Lys)

Start codon

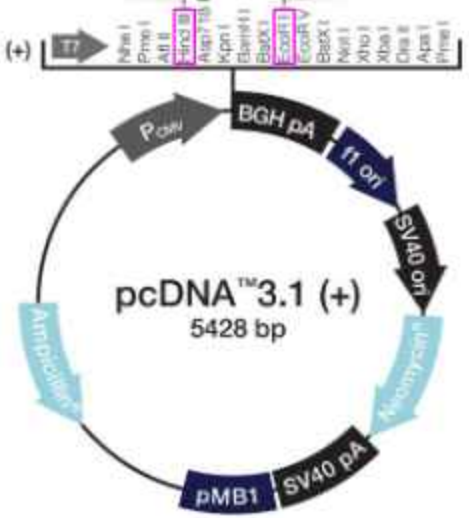
Kozak sequence | Signaling peptide

HindⅢ

AAGCTTGCCACCMNYTSFILAFQLCAILGSSTYYCQAAFFKEIENLKEYFNASNPDVGDGGPLFLDILKNWKEDSDKKIIQSQIVSF
YFKLFENLKDNQVIQKSMDTIKEDLFVKFFNSSTSKLEDFQKLIQIPVNDLKVQRKAISELIKVMNDLSPKANLRKRKRSQNPFRGR
RALQKDHLIHNVHLEEHAHAHNKTAAGAATTC

EcoRI

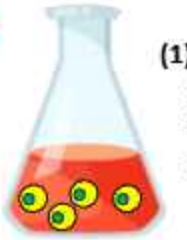
HAT Stop codon



To form the correct structure and improve antibody specificity, eqIFNγ cDNA was inserted into the pcDNA3.1 plasmid and expressed in mammalian cells.

2-1. eqIFNγ-HAT protein expression in mammalian cells (FreeStyle CHO-S cells)

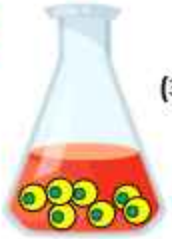
Day0



(1) 24 h before transfection, dilute FreeStyle CHO-S cells to 5–6×10^6 cells/ml and seed into a shake flask.

At 8% CO_2, 37°C, and shaking at 135 rpm for 24 h

Day1



(3) Just before transfection, dilute the cells to 1×10^6 cells/ml.

(2) Dilute plasmid DNA and FreeStyle MAX reagent in OptiPRO™ SFM medium separately, then combine and incubate at room temp. for 15 mins.

Day2

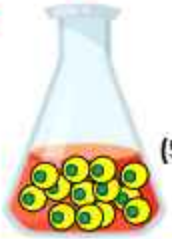
(4) Slowly add 1.2 ml of the DNA-lipid mixture while gently mixing the cells.

Day3

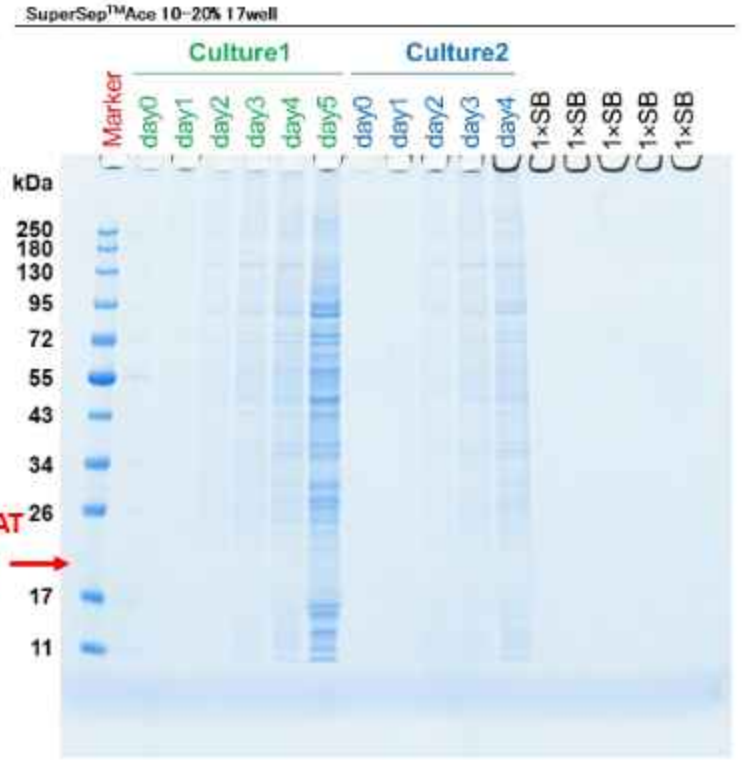
Day4

At 8% CO_2, 37°C, and shaking at 135 rpm for 24 h

Day5

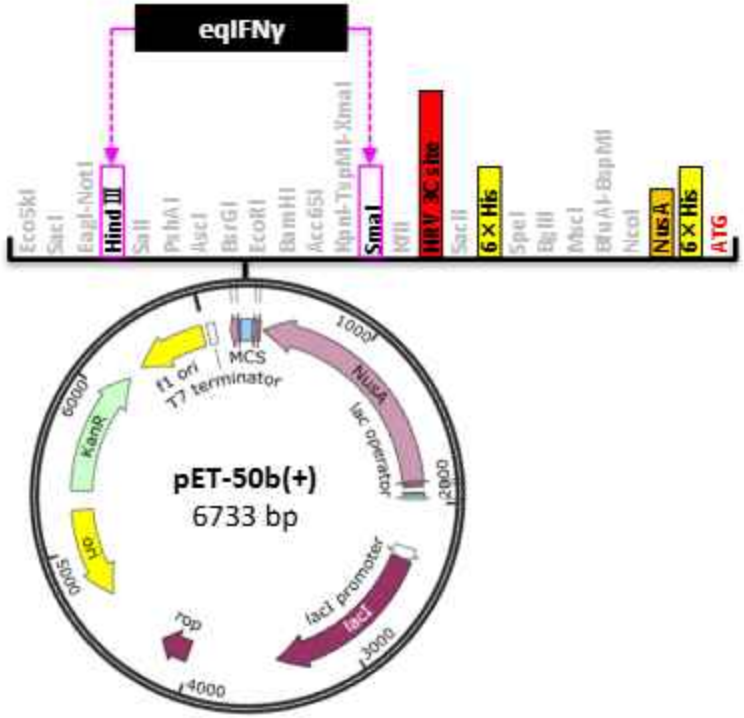


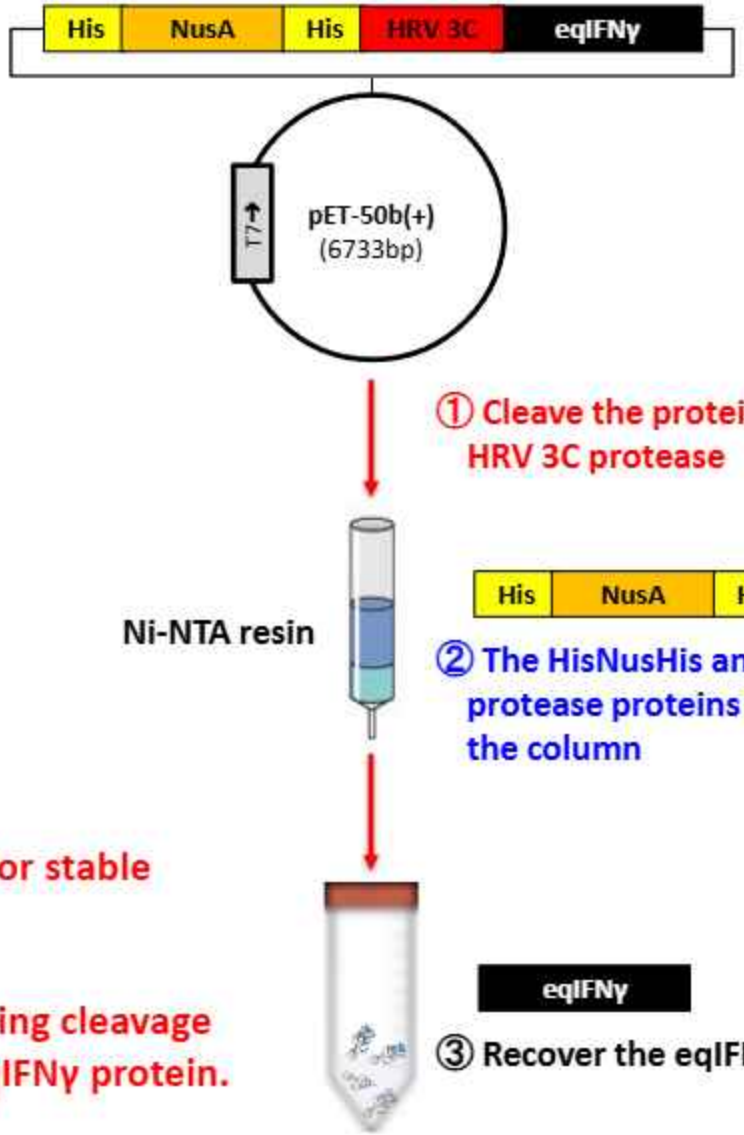
(5) Perform cell counting and sampling every 24 hours until day 5.



The target band was not detected in the SDS-PAGE analysis.

3. eqIFNγ was expressed by inserting it into the pET-50b(+) plasmid





✓ This plasmid contains an NusA-tag, allowing for stable protein expression.

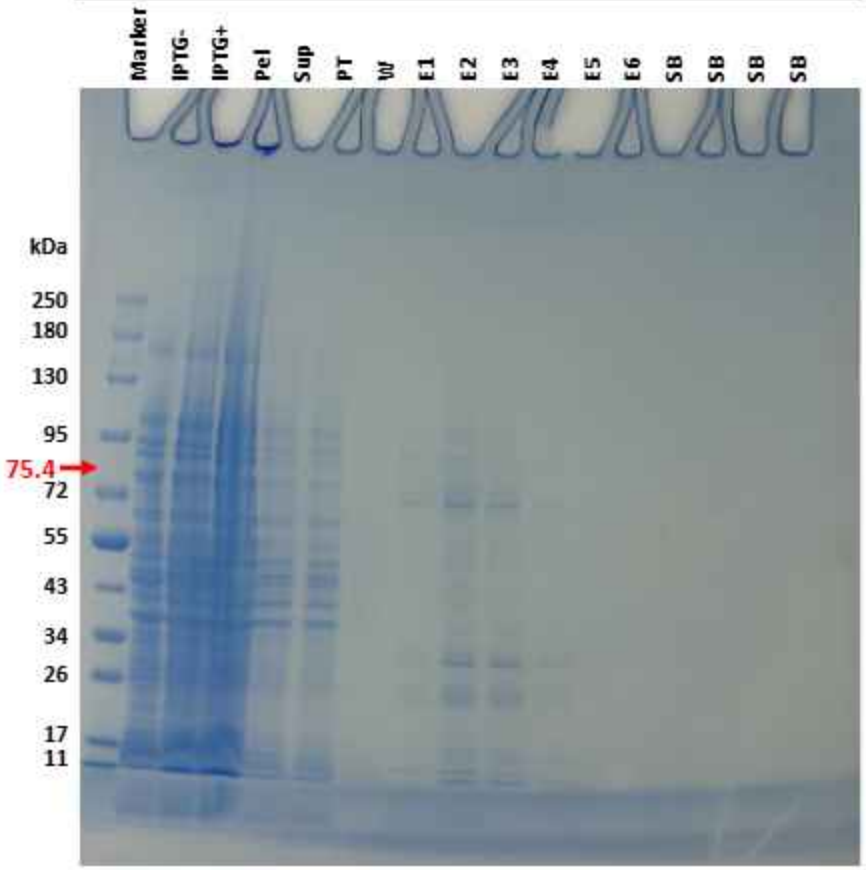
✓ Additionally, it contains an HRV 3C site, allowing cleavage by the HRV 3C protease to recover only the eqIFNγ protein.

3-1. The plasmid was transformed into two types of competent cells, BL21 and Rosetta-gami™ 2(DE3), for protein expression

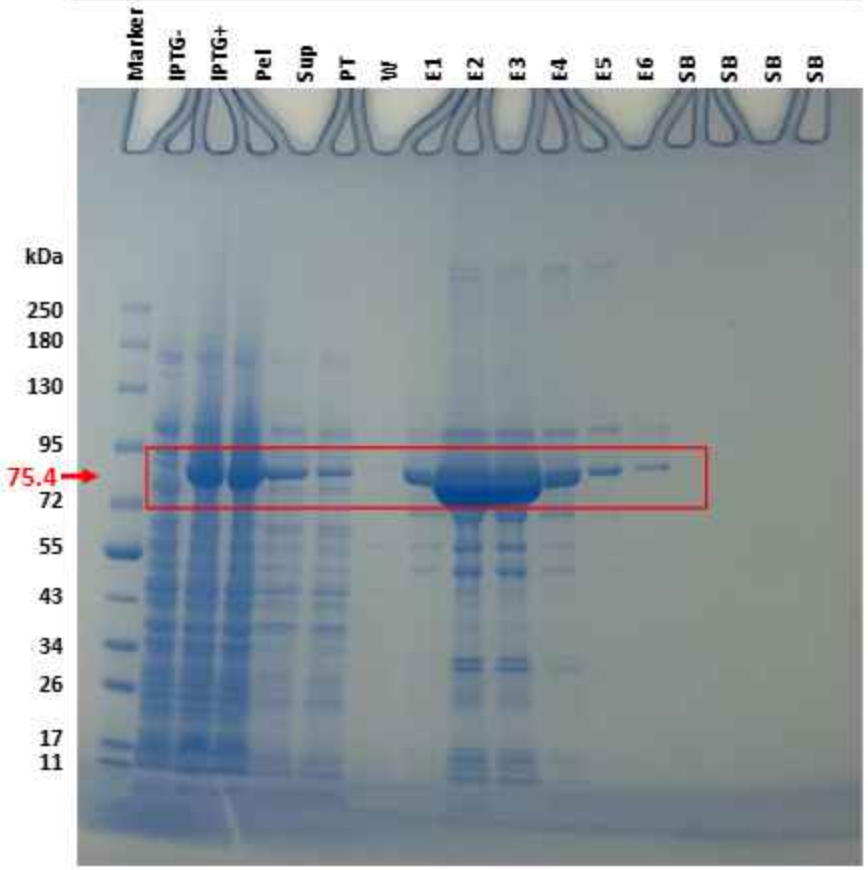
SuperSep™ Ace 5-20% 17well

eIFNγNUS-His ≒ 75.4kDa

eqIFNγNUS-His_pET-50b(+)_BL21

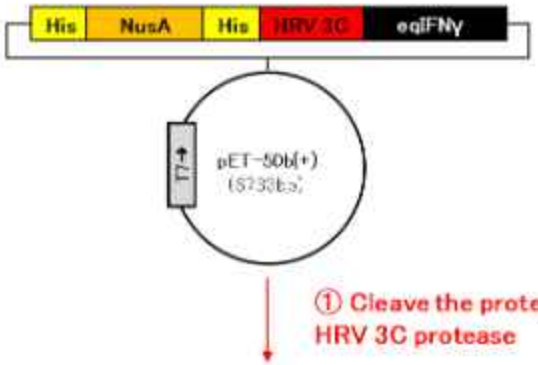


eqIFNγNUS-His_pET-50b(+)_Rosetta-gami™ 2(DE3)



The results showed target protein expression in Rosetta-gami™ 2(DE3), while no expression was observed in BL21.

3-2. Verification of protein cleavage conditions



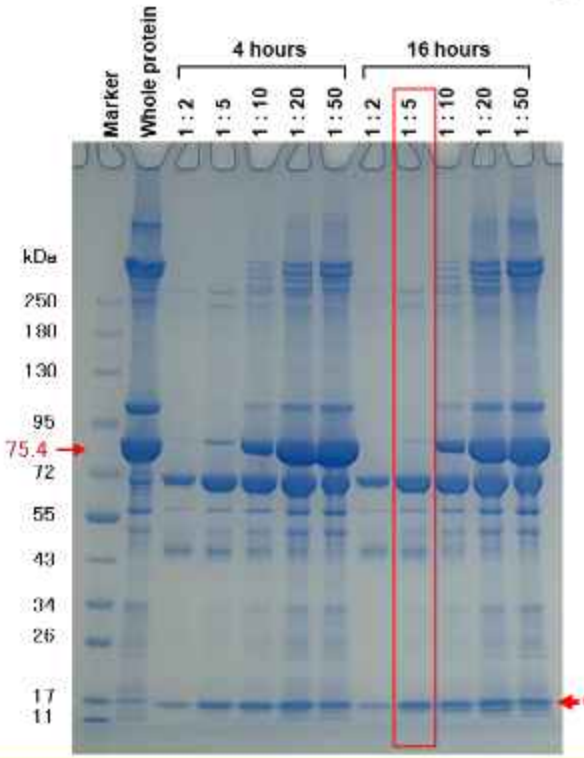
Protein cleavage reaction (**Buffer:** 50mM Tris-HCl (pH8.0), 200mM NaCl)

Protease : Whole protein	1 : 2	1 : 5	1 : 10	1 : 20	1 : 50
Whole protein (ul)	3.6	8.9	17.9	35.7	89.3
HRV 3C protease (ul)	1.0	1.0	1.0	1.0	1.0
Buffer (ul)	45.4	40.1	31.1	13.3	-
Total (ul)	50	50	50	50	90.3

At 4℃, and rotating at 2 rpm for 16 h

※ The samples were collected at 4 hours and 16 hours, respectively, to check the cleavage status

Whole protein conc.: 1.4 mg/ml
Protease conc.: 2.5 mg/ml



The best result was obtained by cleavage at 4℃, with a 1:5 ratio, for 16 hours

Whole protein (eqIFNgNUS-His_pET-50b(+)) ≒ 75.4 kDa;
eqIFNγ protein ≒ 16.7 kDa;
His-NUS protein ≒ 58.7 kDa;
HRV 3C protease protein ≒ 50.3 kDa

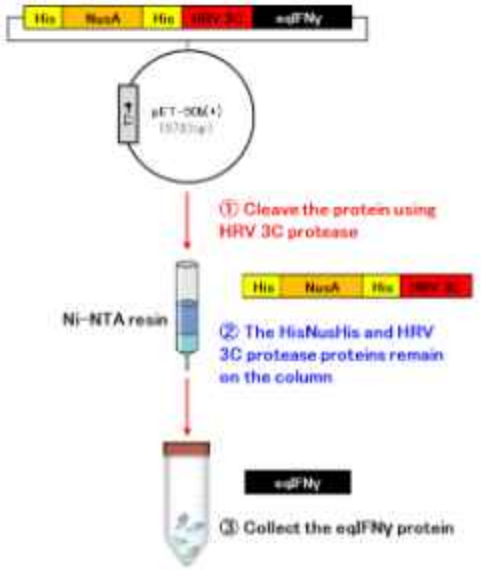
3-3. Separation and purification of cleaved protein by Ni-NTA column

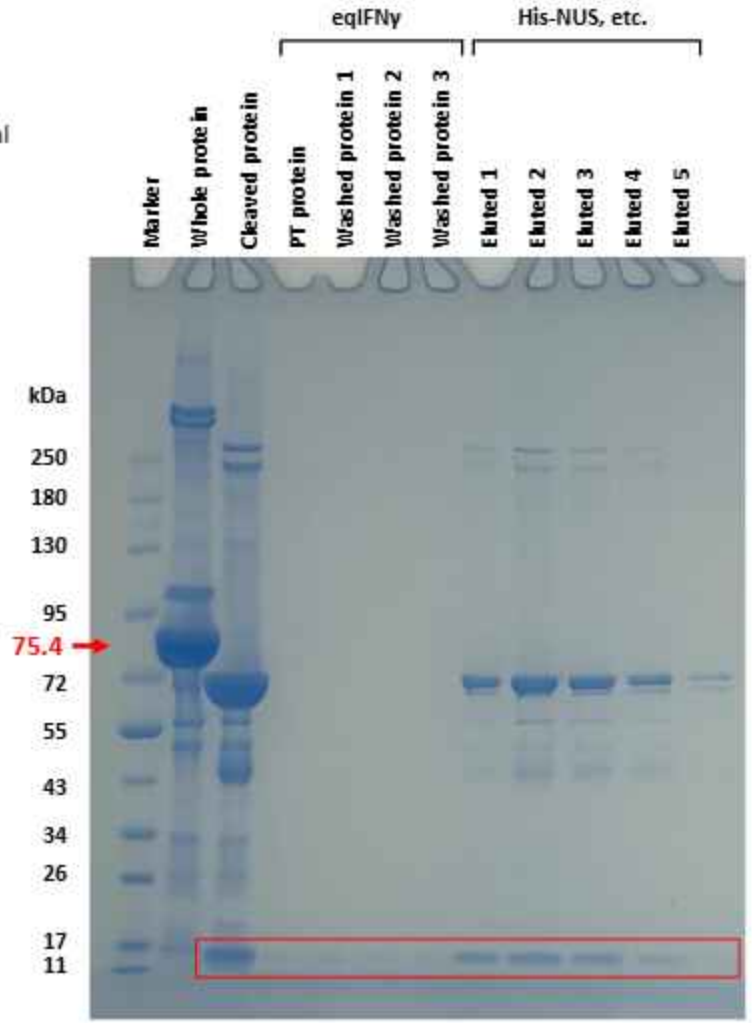
Protein cleavage reaction

Protease : Whole protein	1 : 5
Whole protein (ul)	178.6
HRV 3C protease (ul)	20.0
Buffer (ul)	51.4
Total (ul)	250

Whole protein conc.: 1.4 mg/ml
Protease conc.: 2.5 mg/ml
Buffer: 50mM Tris-HCl (pH8.0), 200mM NaCl

At 4℃, and rotating at 2 rpm for 16 h





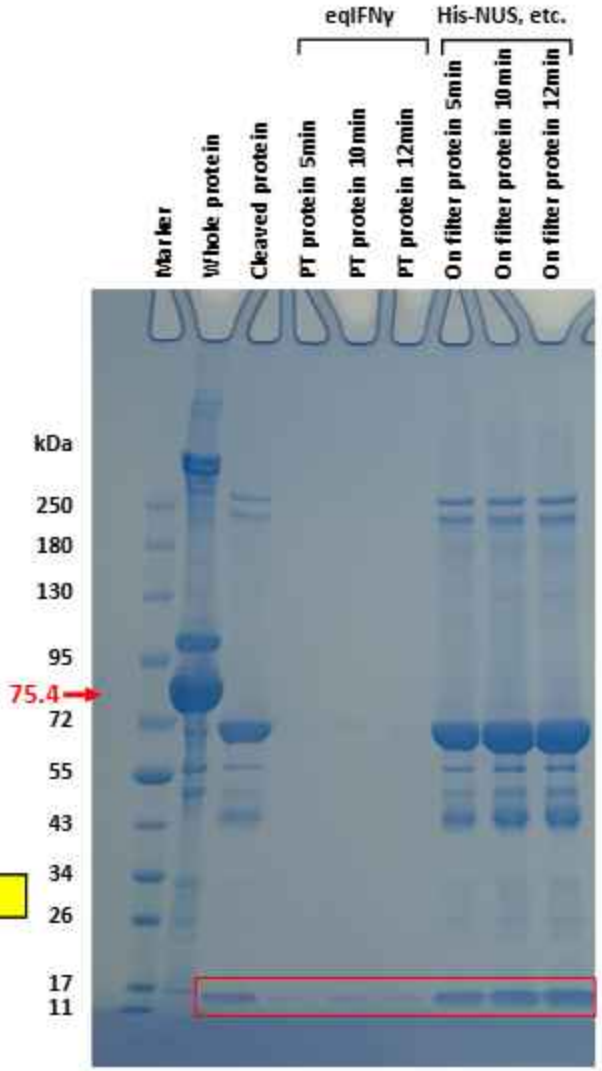
Since His-tags were attached to proteins other than eqIFNγ, these proteins were expected to remain bound to the Ni-NTA column, while only eqIFNγ was supposed to be recovered in the flow-through. **However, eqIFNγ also remained on the Ni-NTA column and was not separated from the other proteins.**

3-4. Separation and purification of cleaved protein by Amicon® Ultra Filter

50k Amicon® Ultra FILTER UNIT

Proteins like His-Nus, being larger than 50 kDa, remain on the filter.

Being smaller than 50 kDa, eqIFNγ passes through the column into the flow-through.



Results: eqIFNγ also remained on the filter and was not separated from the other proteins.

Whole protein (eqIFNgNUS-His_pET-50b(+)) ≒ 75.4 kDa;
eqIFNγ protein ≒ 16.7 kDa; His-NUS protein ≒ 58.7 kDa;
HRV 3C protease protein ≒ 50.3 kDa

3-5. Separation and purification of cleaved protein by four kinds of conditions

Protein cleavage reaction (2 tubes)

Protease : Whole protein	1 : 5
Whole protein (ul)	178.6
HRV 3C protease (ul)	20.0
Buffer (ul)	51.4
Total (ul)	250

Whole protein conc.: 1.4 mg/ml
Protease conc.: 2.5 mg/ml
Buffer: 50mM Tris-HCl (pH8.0), 200mM NaCl

↓ At 4℃, and rotating at 2 rpm for 16 h

1. By denaturing conditions

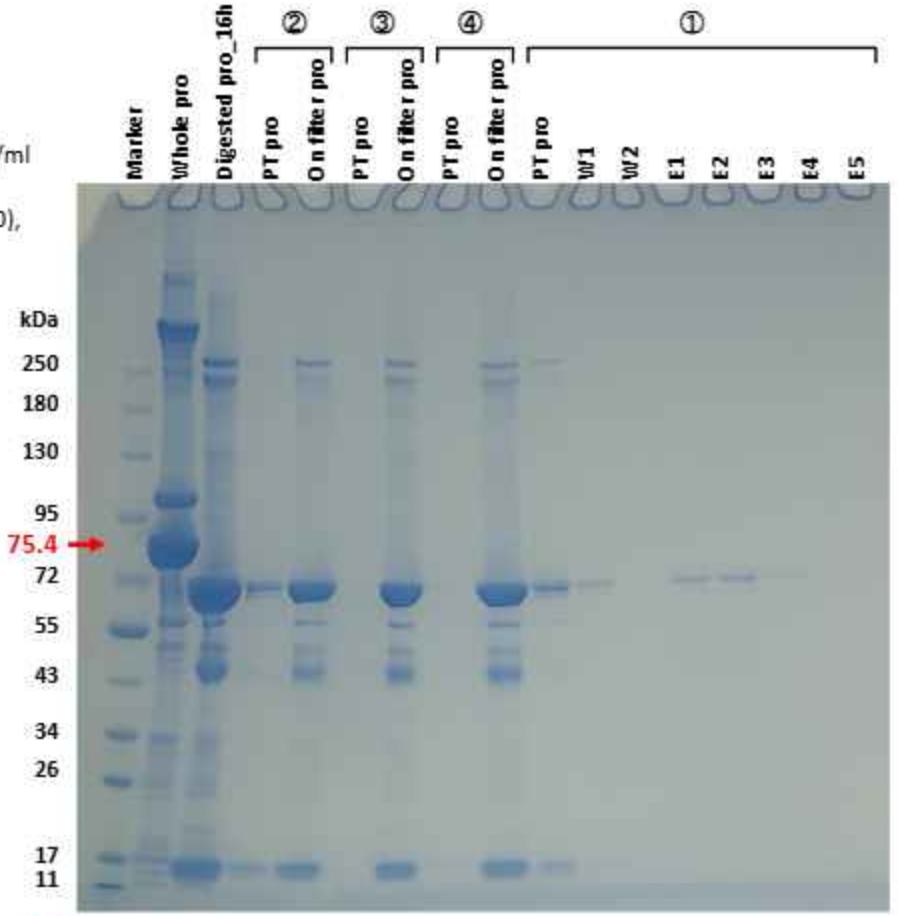
①: Protein + Denaturing buffer + Ni-NTA resin
↓ 4℃, Rotating for 1h
⇒ Purified the protein by Ni-NTA column

②: Protein + Denaturing buffer
↓ 4℃, Rotating for 1h
↓ Filter through an 50k Amicon tube
⇒ Recover the protein

2. high salt concentration condition

③: Protein + 50mM Tris-HCl (pH8.0), 500mM NaCl buffer
↓ Filter through an 50k Amicon tube
⇒ Recover the protein

④: Protein + 50mM Tris-HCl (pH8.0), 1M NaCl buffer
↓ Filter through an 50k Amicon tube
⇒ Recover the protein

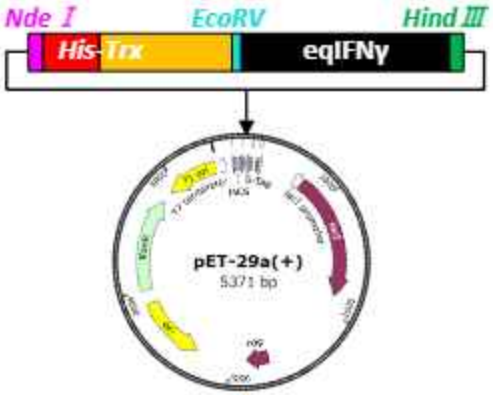


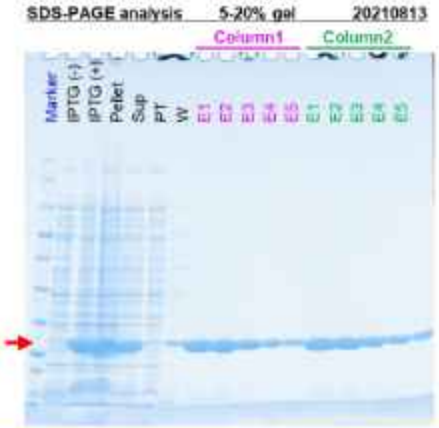
Results: eqIFNγ also remained on the filter or resin was not cleanly separated from the other proteins.

Whole protein (eqIFNgNUS-His_pET-50b(+)) ≒ 75.4 kDa;
eqIFNγ protein ≒ 16.7 kDa; His-NUS protein ≒ 58.7 kDa;
HRV 3C protease protein ≒ 50.3 kDa

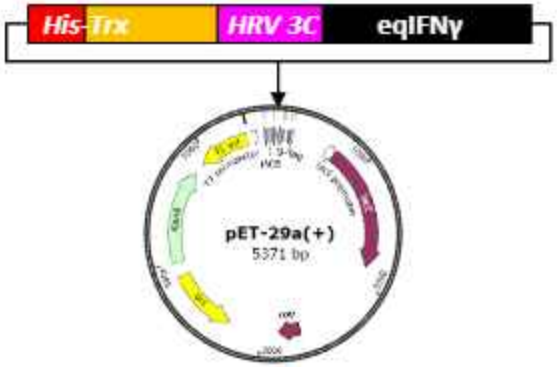
Future Research Plan 1

1. Identify the causes of non-specific binding between proteins and successfully isolate and purify cleaved eqIFNγ.
2. Insert the HRV 3C site between His-Trx and eqIFNγ to express the protein, and then cleave it with the protease to purify eqIFNγ exclusively.



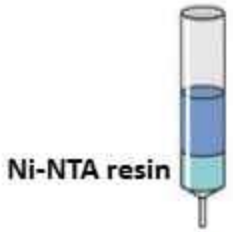


① Synthesize His-Trx-eqIFNγ_pET-29a(+)



② Express the His-Trx-eqIFNγ protein

③ Cleave the protein using HRV 3C protease



※ The His-Trx and HRV 3C protease remain on the column

④ Recover the eqIFNγ protein

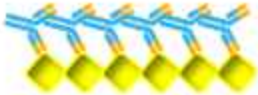
Future Research Plan 2

1. Purification of eqIFNγ-specific antibodies

② Filtration of rabbit serum immunized with the eqIFNγ protein

① Immobilize eqIFNγ protein on Column
HiTrap NHS-Activated HP Column

※ eqIFNγ-specific antibodies remain on the column



③ Elute eqIFNγ-specific antibodies

④ Recover eqIFNγ-specific antibodies

2. Label eqIFNγ-specific antibodies with biotin

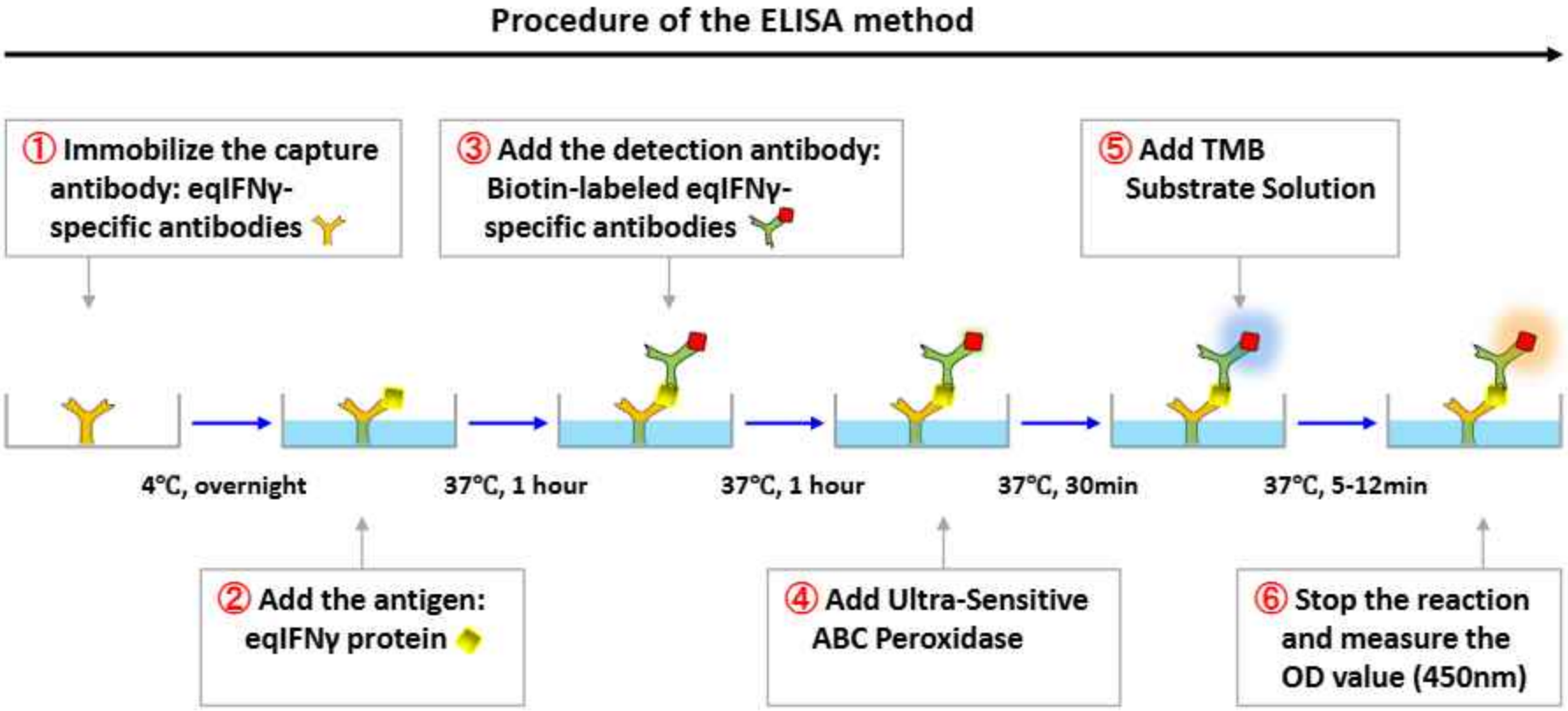
eqIFNγ-specific antibodies

Biotin Labeling Kit-NH2

Biotin-labeled eqIFNγ-specific antibodies

Future Research Plan 2

3. Confirm eqIFNγ-specific antibodies by ELISA



4. Develop the ELISA method

Thank you for your attention