Updated May 2021

This report should be submitted within 2 weeks after you return to Japan. Please do not change the formatting

（Abroad・Domestic） Internship report form （Student） 2022/11/20(Year/Month/Day)

Name	CHEN Yuqi
Laboratory	Laboratory of Molecular Medicine
Year (Grade)	D4
Internship institution	RIKEN Center for Biosystems Dynamics Research
Internship period	Internship period: 11/13/2022 - 11/18/2022 (Departure Date from Sapporo: 11/12/2022, Arrival Date in Sapporo: 11/19/2022)
Purpose	To learn and gain experience in the variable domain of heavy chain antibody, VHH, including synesis, analyzation, basics, and application.

- The reason why you chose this institute

RIKEN is the most famous comprehensive research institute in Japan. Dr. Matsumoto, the host of this internship, is a principal scientist at the RIKEN BDR, with a very broad research area from immunity and virus to protein structure. He is also an alumnus of Hokkaido University, with abundant research and project-leading experience from JAXA to Roche. Now he is focusing on VHH from alpaca in RIKEN. A VHH antibody (or nanobody) is the antigen-binding fragment of heavy chain-only antibodies discovered nearly 25 years ago. In the work so far, VHH antibodies often perform comparably to conventional antibodies for small molecule analysis, are amenable to numerous genetic engineering techniques, and show the ease of adaption to other immunodiagnostic platforms for use in environmental monitoring.

During the discovery of the VHH naturally existing in alpacas, there was cooperation between our laboratory and RIKEN BDR. As my responsibility of arranging and conducting the animal experiment and cell isolation, I also increased my interest in the VHH. Although my research aspect is focused on erythropoiesis, I really would like to expand my research field and become a researcher with a multispectral specialty. During my internship at RIKEN BDR, I believe that I will be able to have an in-depth and extensive exchange with Dr. Matsumoto, not only on the applications and prospects of VHH and nano-antibodies but also on the future development of molecular biology in a more comprehensive and in-depth way.

- Result of the activity （about 800 words、provide photos, tables and figures that clearly show the activities during the period）

Nov. 14 (Mon)

Orientation & E-learning（System for Safety Training）in a.m.

Lecture & Experiment of Cell-Free Fluorescence Protein Synthesis in p.m.

On the first day, I spent a half day on e-learning. Although it's a one-week short internship, Riken still takes it seriously, so I need to complete all the necessary safety training examinations, including gene-editing to chemistry handling.

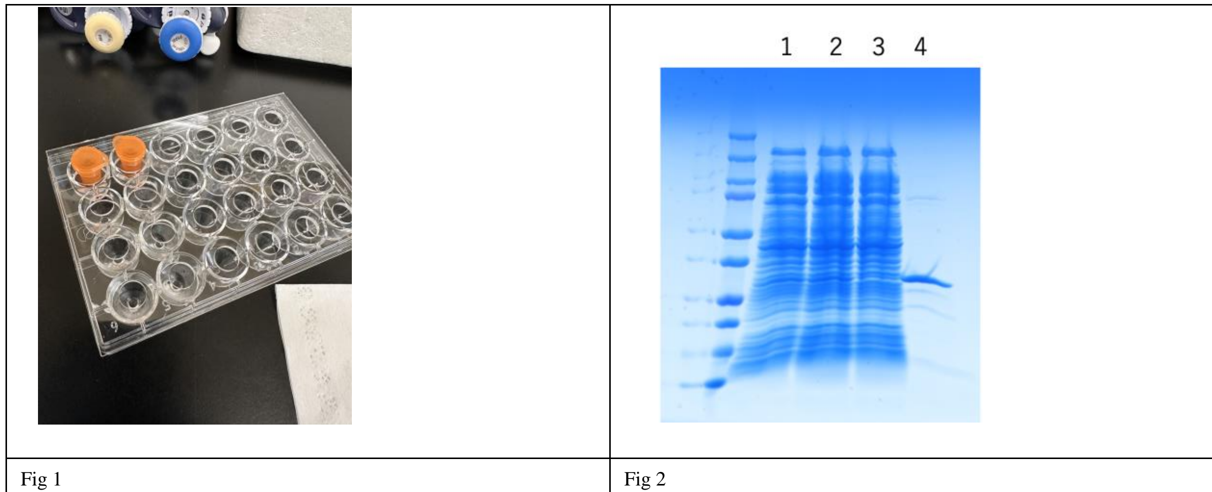

Fig 1 | Fig 2

In the afternoon, I learned about research on the VHH, and the cell-free synthesis system used to produce the antibody protein. Also, I practice using this system to produce GFP protein.

As figure1 shows, in the orange dialysis cup, E-coli S30 extraction, DNA template, and necessary buffer are added. The outer environment only contains a support buffer to ensure the reaction can get enough raw material (ex., amino acid).

The produced GTP protein is infused with His-tag through a TEV sequence. The next step is to purify the protein, and the result is also checked by SDS-page (figure 2). The initial product contains a lot of unspecific proteins (Lane 1). After the high-speed centrifuge and supernatant separation (Lane 2), the protein complex flows through a column with Ni-NTA agarose, which is a His tag specifically binding beads. The wash-through part should not contain GFP protein (Lane 3), and the elution should only contain GFP protein (Lane 4).

Nov. 15 (Tue)

Purification & Evaluation of Synthesized Fluorescence protein & Experiment of Cell-Free VHH Protein Synthesis

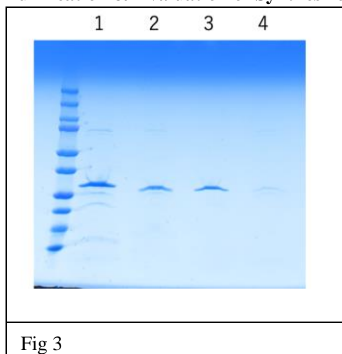

Fig 3

Due to the GFP protein still infusing with His tag (Lane 1), TEV enzyme is used to cleave the His sequence. The protein is incubated with TEV for four hours (Lane 2), then purified by Ni-NTA Agarose. This time, the wash-through part contained GFP protein (Lane 3), and the elution part did not (Lane 4).

Antigen	IgG subtype	KD
hASK1	IgG2	16
hTET1	IgG2	5
hTrx	IgG3	33

With the same procedure, another 3 IgG protein is produced with a cell-free synthesis system. The protein information is listed. All the procedure is similar, and the result is also checked by SDS-page, shown in figure 4 -6.

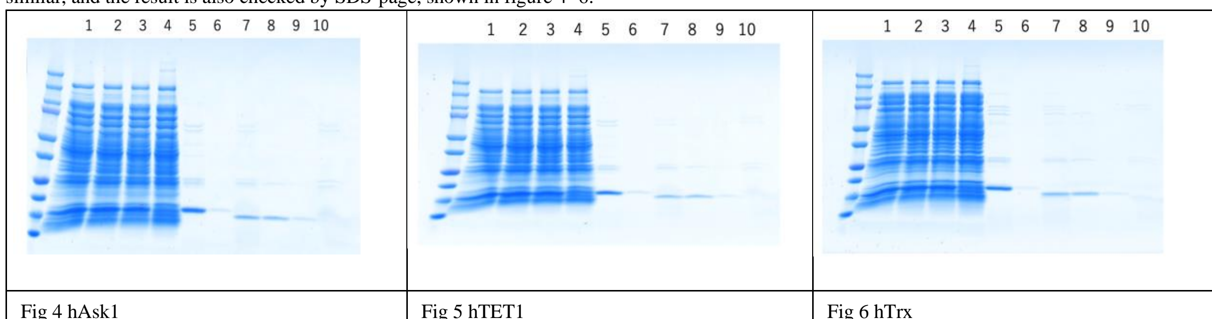

Fig 4 hAsk1 | Fig 5 hTET1 | Fig 6 hTrx

After getting the initial production from the cell-free synthesis system (Lane 1), the supernatant is taken after a high-speed centrifuge (Lane 2). After through a 40nm filter (Lane 3), the protein is loaded on a column with Ni-NTA agarose, washed through part (Lane 4), and elution part (Lane5,6 repeated elution). Also, with the same procedure, the protein is incubated with TEV to cleave the tag (Lane 7) and purified with Ni-NTA agarose. After repeated washing through (Lane 8,9), no target protein was left in the elution (Lane 10).

Traditional cell-based production technologies of antibodies and antibody fragments are well-established. However, these technologies are accompanied by the drawbacks of being rather time-consuming and cost-intensive. As a more efficient and powerful method, cell-free protein synthesis systems have been developed over the last decade as alternatives. Only within 2 days a large amount of protein can be synthesized and easily purified by this method without concerning any cell culture accident.

Nov. 16 (Wed)

Purification of Synthesized VHH Protein & Fluorescence labeling

To observe the portion after transfection, usually, fluorescence labeling is required. Besides designing the infuse protein in advance during the plasmid construction period, a protein labeling kit can also be used to infuse protein directly with fluorescence molecules. The reactive dye has either a succinimidyl ester (SE) or a tetrafluorophenyl (TFP) ester moiety that reacts efficiently with primary amines of proteins to form stable dye–protein conjugates, labeling IgG antibodies or other proteins. Anti hAsk1 is infused with 488, and anti hTET1 with 649. After being infused with a fluorescence probe, the protein is diluted with PBS and concentrated using 10K MWCO protein concentrators 3 times to exchange the buffer. This is the preparation for further cell transfection.

Nov. 17 (Thu)

Administration of Fluorescence-fused antibodies into cultured cells & Introduction of research work and Discussion

To transfect the antibody into the Hela cell, SN21-LK15 intracellular delivery peptide is used. By adding SN21-LK15 to the culture medium along with antibodies, bioactive proteins, and nucleic acids, these biomacromolecules can be efficiently introduced into cells. SN21-LK15 is also expected to be applied to the field of drug discovery as a tool for introducing biomacromolecules into cells. The principle of SN21-LK15 is showed as figure 7.

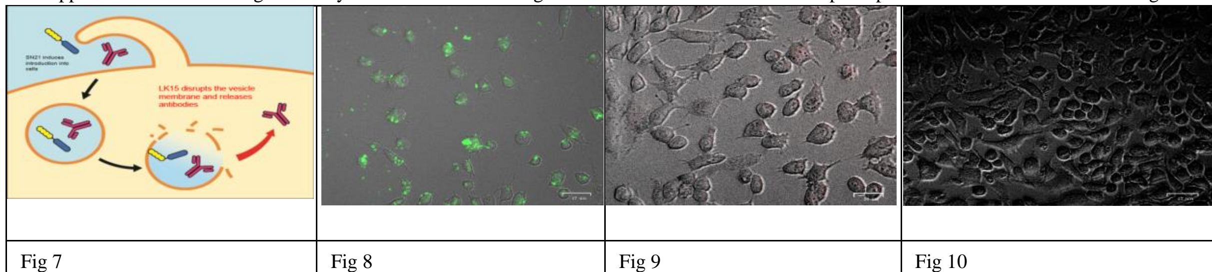

Fig 7	Fig 8	Fig 9	Fig 10

hAsk1 infusing with 488 and hTET1 infused with 649 is shown in figure 8 and 9. Figure 10 shows the negative control. By this transfection method, only 1~2 hours of incubation time is necessary to transfer the target molecule into cells, which is high-efficient, convenient, and budget-saving compared to traditional transfection methods, such as lipo-transfection or electro-transfection.

Nov. 18 (Fri)

Measurement of KD Value of Synthesized VHH against its Antigen by Surface Plasmon Resonance Method using BIAcore

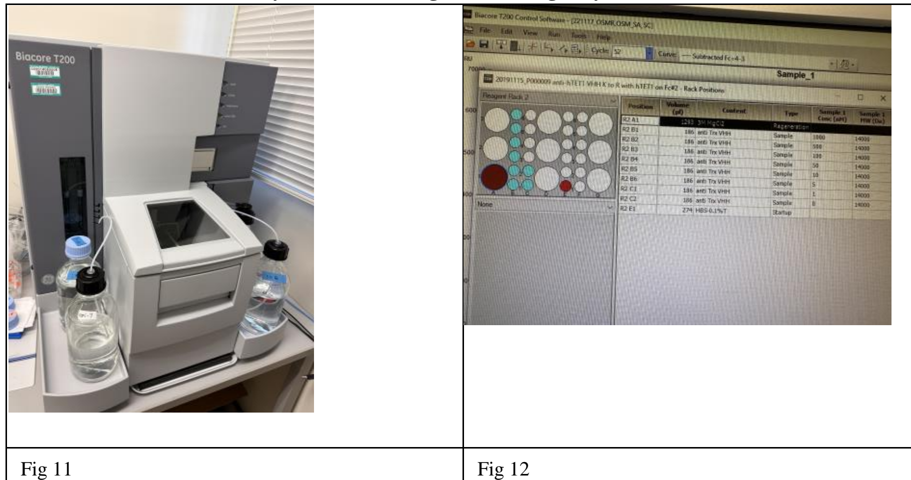

Fig 11	Fig 12

Report table

Curve	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Conc (M)	tc	Flow (ul/min)	kt (RU/Ms)	RI (RU)	Chi² (RU²)	U-value
	6.223E+5	0.02361	3.794E-8	375.2		4.626E+7				3.75	1
Cycle: 6 100 nM					1.000E-7		20.00	1.256E+8	5.059		
Cycle: 7 50 nM					5.000E-8		20.00	1.256E+8	5.202		
Cycle: 8 10 nM					1.000E-8		20.00	1.256E+8	0.4578		
Cycle: 9 5 nM					5.000E-9		20.00	1.256E+8	0.3451		
Cycle: 10 1 nM					1.000E-9		20.00	1.256E+8	-0.9157		
Cycle: 14 100 nM					1.000E-7		20.00	1.256E+8	6.638		
Cycle: 15 50 nM					5.000E-8		20.00	1.256E+8	4.070		
Cycle: 16 10 nM					1.000E-8		20.00	1.256E+8	2.454		
Cycle: 17 5 nM					5.000E-9		20.00	1.256E+8	3.524		
Cycle: 18 1 nM					1.000E-9		20.00	1.256E+8	0.8991		

Table 1

To evaluate the affinity of the antibody, the Surface plasmon resonance (SPR) method is used. A Biacore T200 machine is used, as figure 11 shows. To get the best result, different concentrations of anti-Trx VHH are arranged on the rack (Figure 12). The result is showed in table 1. Chi^2 indicates the reliability of the result. Usually, the Chi^2 should be below 20, which can be affected by the concentration of the sample. The result shows that the KD value of this sample is 37 nM, which is quite close to the theoretical value. KD is the equilibrium dissociation constant, a ratio of koff/kon, between the antibody and its antigen. KD and affinity are inversely related. The KD value relates to the concentration of antibody (the amount of antibody needed for a particular experiment) and so the lower the KD value (lower concentration) and thus the higher the affinity of the antibody.

- What do you think the positive impact of the activity will have on your other career path?

During this one-week internship, I took part in the whole process of a cell-free protein synthesis system, including the following purification and verification. This experience expanded my knowledge of biotech and got me in touch with the aspect which is far from my research field, especially immunology and bioengineering. As my career plan is to work in a life science company, expanding my biotech knowledge is quite important. At Riken, I've learned several leading-edge experiment methods and understand the industry trends in life science, including CAR-T and other aspects. Also, I visited the public laboratory in Riken with many advanced instruments, including NMR and cryogenic electron microscopy. It's a pity that I didn't have a chance to learn or use these instruments, neither allowed to take photos.

This internship showed me the forefront of life science and gave me an up-to-date understanding of the biotechnology industry. This will be of great help to me in my future career path.

- Advice for your junior fellows

For a Ph.D. student with limited connection, there is no need to hesitate to ask for help from your supervisors. Establishing an interpersonal relationship is one of the purposes of the Ph.D. course. With the help of a senior researcher, it's more easily for you to get in touch with an ideal internship opportunity.

Approval of supervisor	Institution・Official title・Name Laboratory of Molecular Medicine・Professor INABA Mutsumi

※1 Send the electronic file to the WISE Program Office

※2 Attach a copy certificate of the content of internship activity that is prepared by the counterpart at the internship institution (any form with a signature of the counterpart).

※3 The Steering Committee for the WISE Program will first confirm the content of this report and report will be forwarded to the Educational Affairs Committee for credits evaluation.