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ORIGINAL ARTICLE

# Characterization of Produced Novel Nanobody Against CD19 Marker

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## ABSTRACT

**Objectives:** The engineered single domain antibodies, which produced against CD19 immune receptor, distinguished by many properties

**Methods:** Here, a set of characteristics were described, including protein expression of VHH gene in positive transformed Er2738 colonies VHH gene expression was detected by Real-Time PCR (RT-PCR) so threshold cycle Ct was 34.9, purification of soluble nanobodies' protein carried out in which the fractionation of the dissolved nanobodies protein from other periplasmic proteins using a gel chromatography by Sephadex LH-G20.

**Results:** The resulting curve illustrated a unique peak with the highest optical density (OD) of 280nm was 0.58. The specificity of soluble nanobodies against CD19 at 37°C and 80°C were determined by Enzyme-Linked Immunosorbent Assay ( ELISA).

**Conclusion:** The results demonstrated characteristics feature of nanobodies' ability to bind specifically with the interested antigen at a temperature of 37°C and continued their effectiveness at 80°C, which is the diagnostic characteristic of nanobodies.



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## INTRODUCTION

Single domain antibodies (SdAb), also named Nanobodies (Nbs), are derived from a unique type of antibodies restricted to their presence in members of the camelid family and sharks.[1] These antibodies are called heavy-chain antibodies (HcAb). The natural discovery of HcAbs, followed by the development of recombinant Nbs, represents an attractive alternative in the therapeutic application, diagnosis, and drug delivery.[2,3] Nbs particular interest because of its important features, especially one unique aspect as thermostability because it can have immediate effects on conformational stability.[4] Most studies refer to the optional disulfide bonds eliminated aggregation of Nbs that gives further effect represented by increasing length of complementarity determining loop which, expected this configuration gives heat resistance.[5] Expression of the recombinant protein in prokaryotic system, especially in E. coli proved wide success due to simple, easy work, rapid growth, moderate price medium, and the capacity to produce recombinant protein about 50% of total cellular protein.[6] While the protein synthesis in prokaryotic is faster than eukaryotic, approximately ten times more, but this makes that this expression system is inefficient due to the slow folding leading to more accumulated recombinant proteins[7]. The aggregation of both Unfolded and folded are due to exposure to hydrophobic surface, and some recombinants proteins can fold in vivo with an accessory protein named chaperons.

The bacterial extract has been a convenient means for in vivo production of recombined protein because they have the entire machinery required to synthesize proteins,[8] so picked a single colony to start the fermentation process by *E. coli.*

Fermentation produces 50–80% g/of cell weight to generate 2–5% of the total cellular protein, meaning 100–300mg of recombinant protein is formed in the host cell.[9]

Eukaryotic protein expression in E. coli often leads to accumulation of insoluble protein either folding or unfolding, known as inclusion bodies, dense amorphous bodies and can be observed by phase-contrast microscope and isolated by centrifuge.[10] The synthesis recombinant protein may be observed in the cytoplasm or direct to periplasm or secreted to culture medium by the cell itself[11] inclusion bodies can protect the proteins from proteolytic reaction and permit protein accumulation naturally toxic to the host cell.[12] Protein aggregation causes lead to a decrease in production, and many strategies were offered in biotechnology research to increase insoluble proteins in vitro, so Protein purification is a process designed to separate a single copy from the complicated mixture.

## MATERIALS AND METHODS

The Positive transforming bacteria that carried the recombinant gene. (VHH–pComb3XSS) fragment[13] cultured on LB (Luria-Bertani) media with glucose 0.4% as fermentation media induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) for protein expression performed.[14]

For Expression of Nbs gene in transformed *E.coli Er2738,* Quantitative Real–time PCR (qRT-PCR) (Intron bio/ Korea) was used, RNA was extracted from positive bacterial colonies carrying the recombinant gene. Using AccuPower® RocketScript™ RT PreMix kit™ from Bioneer according to manufacturer's instruction. The RNA samples were converted to cDNA using AccuPower® RocketScript™ RT PreMix kit intron Biotechnology Company.

Real-Time PCR reaction done according to procedure In AccuPower® GreenStarTM qPCR PreMix tube, the following materials were added as in Table 1, while the primer designed using amplification illustrates in Table 2 and the condition of reaction in Table 3.

The expression of recombinant single domain antibodies (nanobodies protein) was accumulated in periplasmic space of E.coli fractionated of single domain antibodies protein from other bacterial proteins by gel chromatography, the Sephadex (LH 20) used for purification of protein extract into molecular size according to the method.[15] For confirming the specify and activity of produced soluble anti CD19 Nbs was done by Enzyme-linked Immunoassay (ELISA). ELISA kit (Santa-Cruz/USA) was used.[16] Using fractionated protein extract at 37°C, the efficacy of Nbs was tested, and the specificity of the binding persisted at high temperatures where the protein extract-treated at 80°C. The result was detected by ELISA reader (450nm).

Table 1. **The contents of PCR tube and their volumes**.

| Competent | Volumes /µl |
|---|---|
| **Master mix** | 10 |
| **Forward primer** | 1 |
| **Reverse primer** | 1 |
| **cDNA templet** | 2 |
| **N.F.W** | 6 |
| **Total** | 20 |

Table 2. **The primers of Real-Time PCR reaction.**

| Name of primer | Sequences | Tm |
|---|---|---|
| **VHH qr1** | ACGGACTGGGAGTATCCACT | 62 |
| **VHHqF1** | ATCCTTCACCAGGCAAGCAA | 60 |

## RESULTS

Expression of VHH gene was detected via RT-PCR. Results displayed the presence of VHH gene in the selected clones in the prepared Cdna (Figure 1). The reaction was subjected to melting curve analysis in terms of primers specificity and detection of one specific product. The primers gave one single, distinctive peak at 78°C (Figure 2).

Soluble Nbs were purified from other soluble periplasmic protein by gel chromatography using Sephadex LH-G20 (Figure 3) shows absorbance values of the fractions. It was noticed that the absorbance increased gradually for each fraction until it reaches the maximum concentration (fractions 5,6); the highest OD was 0.58 for fraction 5.the concentration of purified Nbs was measured to be 0.50 according to a standard curve of BSA (Figure 4).

ELISA measures the soluble Nbs for their binding capacity to the CD19 antigen. Both selected Nbs have confirmed their specificity to the CD19 protein, ELISA assay was managed with serial dilutions of soluble antiCD19- Nbs. Achieving efficient binding results with the interested antigen; the highest

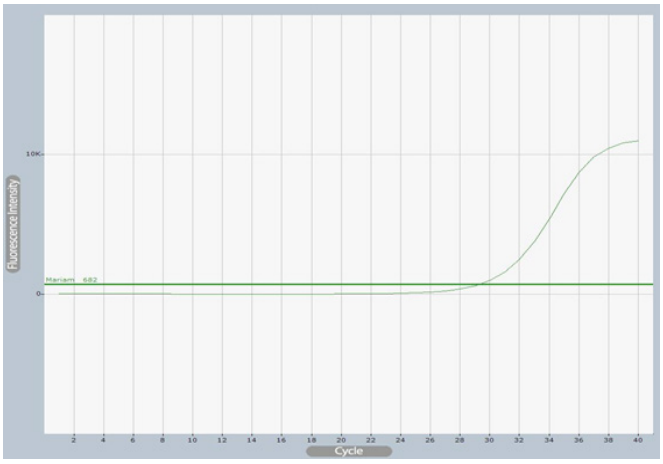


Figure 1. **VHH amplification curve. A VHH amplification curve represents the machine-set threshold automatically, and the signal was detected at the typical CTs range.**

Table 3. **The Real-Time PCR program.**

| NO. | Steps | Temperature | Time | NO. of Cycles |
|---|---|---|---|---|
| 1. | Denaturation | 94 | 3 | 1 |
| 2. | Denaturation | 94 | 30 | |
| 3. | Annealing | 60 | 30 | 40 |
| 4. | Extension | 72 | 1 | |
| 5. | Extension | 72 | 5 | 1 |

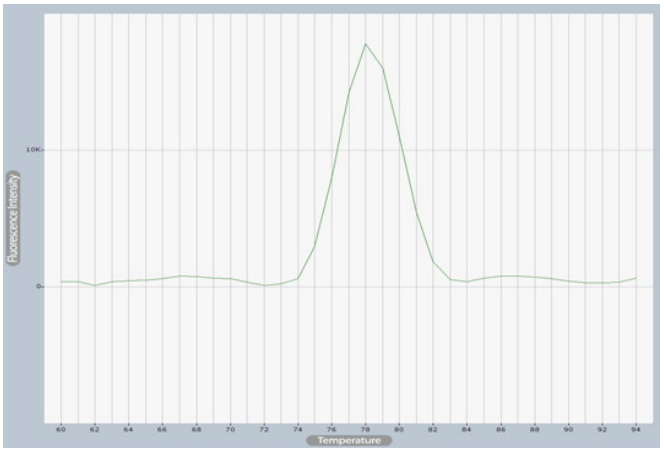


Figure 2. **Melting peak of VHH gene expression**

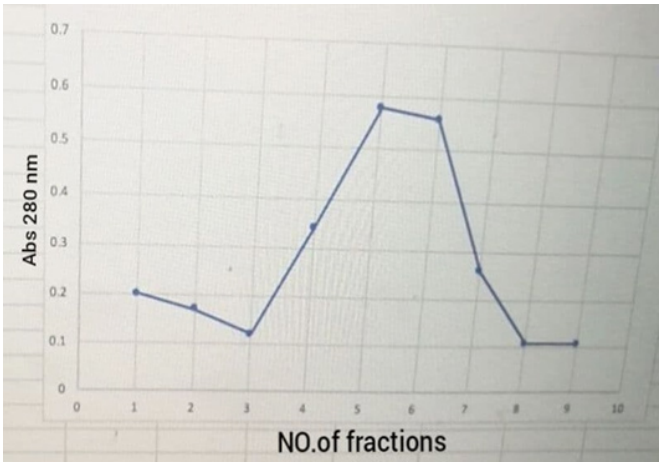


Figure 3. **Absorbance values of proteins solutions for purification of soluble Nbs.**

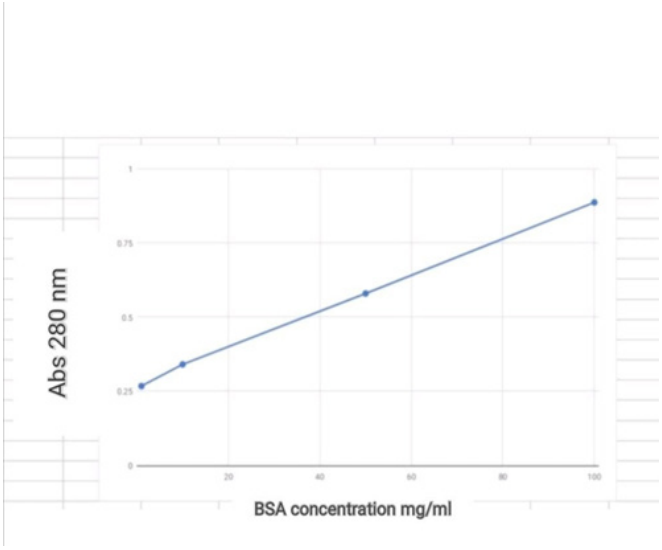


Figure 4. **Standard curve of Bovine Serum Albumin (BSA) as a standard protein according to Lowry method. The concentration of soluble Nbs (Fraction, 5) was determined to be 0.5 mg/mL.**

O.D/450nm was 3.5 at dilution 1:1 whereas the OD was 2. 279 in 1:2 the OD were 2.279 and 1.825 at a dilution rate 1:3 and

| | | | 37°C | | | 80 °C | |
|---|---|---|---|---|---|---|---|
| DILUTION | O.D | 1 | 2 | 3 | 4 | 5 | 6 |
| 1:1 | A | 0.124 | 3.5 | 3.5 | 3.5 | 3.452 | 1.327 |
| | B | 0.127 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| | AV | 0.125 | 3.5 | | | .2944 | |
| 1:2 | C | 0.098 | 2.351 | 2.374 | .2205 | 1.994 | 1.831 |
| | D | 0.107 | 2.152 | 2.211 | 2.381 | 1.686 | 1.894 |
| | AV | 0.1025 | 2.279 | | | .1851 | |
| 1:3 | E | | 1.866 | 1.830 | 1.988 | 1.660 | 1.553 |
| | F | | 1.799 | 1.729 | 1.752 | 1.535 | 1.565 |
| | AV | | 1.825 | | | 1.578 | |

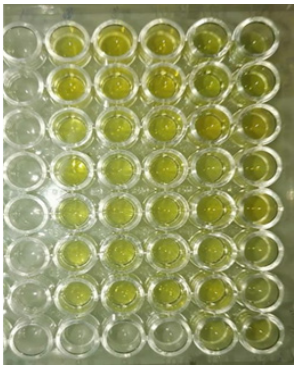

Figure 5. **ELISA reaction test plate to clarify the specificity and activity of Nbs towards soluble CD19 antigen plate outline as following: Column 1: (A, B) control PBS, (C, D) Blank. Columns 2,3,4: ELISA OD/450nm value measurement the specificity at 37°C. Columns 5,6: ELISA OD/450nm value measurement the binding efficiency continues with specificity at a temperature of 80 °C.**
*** Aliquot of every dilution rate was referred by the same color.**

1:4 respectively the control (PBS) had no sign of binding to the target. The study demonstrated the continued effectiveness of Nbs to bind efficiently even at high temperatures (80°C). The highest OD/450nm was 2.944 at dilution1:1, whereas the OD/450 were 1.851,1.578 at dilution 2:1 and 3:1, respectively, as shown in Figure 5.

## DISCUSSION

Detection of the VHH message indicates the success of transformation at the mRNA level. Quantitative PCR has many advantages, such as accuracy and sensitivity. The quantification reaches 8 logs of magnitude and 100000 sensitivity, compared to RNase protection assays, respectively. The qPCR can detect a single copy of the transcript. Finally, the real-time PCR enables the researcher to discriminate between the almost identical mRNA sequences.

Nbs can either expressed within the cytoplasm or directed toward periplasmic space of prokaryotic system using bacterial leader Signal. Periplasmic extracts are preferred.[17] It is equipped with a good environment for folding and oxidative environment for disulfide bond synthesis. Moreover, periplasmic expression considers as the easiest and most efficient with minimizing bacterial protein contamination using a simple osmotic-shock method, which makes the purification process very straightforward.[18]

in the present study, Sephadex LH-G20 column chromatography was used to purify soluble Nbs from other soluble periplasmic proteins; this process considers an important step for evaluating their interaction with interest antigen.[19]

The purification of Nbs by column chromatographic gel was also preferred by more than one strain of *E. coli* (BL21 (DE3), Rosetta-gami B (DE3) pLysS, Origami 2 (DE3), and SHuffle T7) was used and displayed efficiency results. Hence, *E. coli* was used in the current study for expression system, and column chromatography technique was carried on for purification.[20,21]

## CONCLUSIONS

The single-domain antibodies are generally well expressed at a low cost with a high degree of stability and solubility in the prokaryotic system.

The ability of these Nbs to give sharp results in ELISA assay after a heat shock to 80 adding a positive characteristic can withstand high temperatures without losing its specificity to interact with the antigen for which it produced.

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