Enhancement of a Humanized Nanobody against HER2 Tyrosine Kinase Production in the Cytoplasm of *Escherichia coli*

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***Abstract*—Around 30% of invasive breast cancers are associated with the overexpression of the human epidermal growth factor receptor 2 (HER2) protein. While monoclonal antibodies are commonly used in targeted therapies, their large size hinders efficient delivery to tumor cells. Nanobodies, single-domain antibody fragments, provide a promising alternative due to their high binding affinity and small size. VHH17, a nanobody specific to HER2-Tyrosine Kinase (HER2-TK), blocks phosphorylation and reduces cancer cell proliferation. This study aimed to develop a humanized anti-HER2-TK nanobody and optimize its production in the cytoplasmic space of *E. coli*. The effects of temperature and induction on soluble expression were evaluated. Western blot analysis revealed that 20 °C and 1 mM IPTG induction were optimal for humanized VHH17 expression. These results demonstrate that *E. coli* can effectively express humanized VHH17 under proper conditions. Further optimization may enhance the yield of soluble VHH production.**

***Keywords*—antibody fragment expression, *Escherichia coli*, human epidermal growth factor receptor 2 (HER2), tyrosine kinase, VHH single-domain antibody**

I. Introduction

Currently, breast cancer is the most prevalent cancer in adults, with an annual incidence of over 2.3 million cases. It stands as the foremost or second leading cause of female cancer-related deaths in 95% of countries worldwide [1]. Approximately 30% of invasive breast cancers overexpress the human epidermal growth factor receptor 2 (HER2) protein. Targeted cancer therapy can be achieved using monoclonal antibodies and kinase inhibitors; however, with the large size of monoclonal Antibodies (mAbs), challenges are present in efficiently delivering them to endogenous tumor cells. Nanobodies (Nbs), also known as VHHs (variable heavy domain of heavy chain antibodies) are single-domain antibody fragments derived from the variable domains of Camelidae Heavy-Chain-only Antibodies (HCAbs). They offer a potential solution to overcome these limitations. Over the past three decades, they have gained widespread use in various research fields and have attracted interest from companies for their potential applications in diagnostics and therapeutics. Nbs offer several advantages over mAbs. Their unique structure, characterized by their small size, convex shape, and extended Complementarity Determining Region 3 (CDR3), allow them to bind to concave sections of antigens that are often inaccessible to the larger mAbs. Despite their minute size, Nbs maintain high binding affinity, comparable to other mAbs. The smaller size of Nbs also allows for improved tissue penetration, enabling specific and efficient drug delivery, as well as enhanced imaging capabilities. Moreover, Nbs possess remarkable stability when exposed to high temperatures and remain soluble, stable, and resistant to proteases and pH changes. They also exhibit low immunogenicity, making them promising candidates for drug development with reduced risk of adverse reactions during clinical trials. Additionally, Nbs can be easily modified, produced, and purified through various expression systems, allowing for economical production for diagnostics and therapeutics with potential broader antigen recognition than mAbs [2].

The potential applications of Nbs for diagnostic and therapeutic purposes are significant. Nb-based agents have shown promising anticancer activity, especially in HER2-positive breast cancer treatment. Lamtha *et al*. [3] recently identified VHH17, an Nb specific to the HER2-tyrosine kinase (HER2-TK) domain, using phage display. VHH17 can block kinase phosphorylation, thus reducing cancer cell proliferation and disease progression.

Recombinant Nbs are commonly produced by secreting into the *E. coli*'s periplasm, where the oxidizing environment supports disulfide bond formation, thereby stabilizing their structure. After secretion, Nbs can be recovered from the supernatant following an osmotic shock. On the other hand, the reducing environment of the bacterial cytoplasm limits disulfide bond formation, though some Nbs can fold functionally without these bonds. In the past 30 years, mutant *E. coli* strains with an oxidizing cytoplasm, such as Rosetta-gami B (DE3) and SHuffle® T7 cells, have been developed to enhance the expression of disulfide-dependent proteins [4].

For therapeutic purposes, the amino acid sequences that are specific to Camelidae family in the framework must be modified to match their human heavy chain variable domain equivalent, i.e. humanized. A general strategy to humanize Nbs based on a Camelid single-domain antibody has been

reported by Vincke *et al*. (2009) [5]. They carefully analyzed camelid VHH compared with human heavy-chain-variable region (VH) and identified a soluble, stable, and highly expressed universal humanized Nb scaffold, h-NbBcII10FGLA, that enables the grafting of antigen-binding loops from other Nbs allowing the grafted Nbs to retain both antigen specificity and affinity.

In this study, we humanized anti-HER2-TK reported by Lamtha *et al*. (2021) [3] by grafting its antigen-binding loops onto the h-NbBcII10$_{FGLA}$ scaffold. Non-optimal codon usage in heterologous protein expression can reduce translation efficiency and protein levels due to limited availability of cognate tRNAs in the host. Therefore, we utilized codon optimization strategy as well as explored several *E. coli* strains and protein expression conditions to enhance the production of this humanized Nb in the cytoplasm of *E. coli*.

II. Materials and Methods

A. Construction of VHH17 for Cytoplasmic Expression

The amino acid sequence of the original VHH17 was provided by our collaborators at Kasetsart University. The genes of both the original and humanized VHH17 were optimized for *E. coli* expression and chemically synthesized by GenScript, Inc. (USA) in a pUC19 plasmid vector. The VHH17 gene fragments were amplified with flanking NcoI and SalI cut sites for cloning into the pET-28(a) vector (pET28a-scFv-GCN4), which includes a C-terminal FLAG tag and a 6x-His tag [6]. This process resulted in the creation of the pET28a-VHH17 vector, also containing a C-terminal FLAG tag and a 6x-His tag.

B. Bacterial Strains and Growth Conditions

For cloning, *E. coli* strain NEB10β (New England Biolabs, USA) was used. For VHH17 expression from pET28(a) vector, various strains of *E. coli*, i.e., BL21(DE3), MC100(DE3), SHuffle® T7 Express (SF(B)), SHuffle® T7 (SF(K12)) were used. Plasmids were transformed into each strain using electroporation technique. For protein expression, cells were grown overnight in LB medium containing 50 µg/mL kanamycin (Kan) at 37 °C with 200 rpm agitation in an incubator. The next morning, each overnight culture was normalized in fresh LB medium until the optical density at 600 reached 0.6, after which protein expression was induced by adding 0.05 mM, 0.5 mM, or 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were then incubated at 20 °C, 30 °C, or 37 °C with 200 rpm agitation.

C. Protein Analysis Using Western Blot Analysis

Cells expressing VHH, using various conditions described in section B, were harvested 24 hours after induction by centrifugation at 10,000 RCF for 10 min at 4 °C, adjusted to an OD_{600} of 50, and resuspended in 300 µL of Phosphate-Buffered Saline (PBS). The samples were sonicated on ice using a Sonifier® SFX150 (Branson, USA) for three 30-second intervals at 45% amplitude and a 50% duty cycle, followed by centrifugation at 10,000 RCF for 10 minutes at 4 °C. The supernatant was collected as soluble proteins. To denature the soluble proteins, samples were mixed with 6x loading dye mixed with β-mercaptoethanol and boiled at 100 °C for 10 min to denature the proteins. For western blot analysis, 20 µL of soluble protein samples normalized by OD_{600} as mentioned above were separated using 10% polyacrylamide gels (TGX FastCast Acrylamide Solutions, Bio-Rad, USA) and transferred onto polyvinylidene fluoride (PVDF) membranes. Western blotting was performed according to standard protocols. The membranes were probed with 1:3000 dilution of mouse anti-FLAG M2-HRP antibodies to detect the FLAG-tagged Nb [6].

D. Relative Quantification of VHH Expression and Growth Conditions

The Image Lab 6.1 program on the Bio-Rad ChemiDoc™ MP Imaging System (USA) was used for densitometric analysis of the protein bands. Relative solubility was calculated by comparing the expression of each sample with that of the reference sample, which is the sample from cells expressing VHH17 at 20 °C with 0.5 mM IPTG induction. This condition was used as the reference because it was employed for Nb expression in many previous studies [7–10].

III. Results and Discussion

A. Humanization of VHH17 Nanobody

The original VHH17 was obtained from a humanized VH/VHH phage display library. This Nb was isolated using a single round of bio-panning. It showed high specificity, affinity, and kinase inhibition activity against HER2-TK. The fusion of cell-penetrable peptide (R9) to VHH17 Nb allowed cell-penetrating ability resulting in HER2-positive cancer cell growth inhibition by targeting the ATP-binding pocket, blocking HER2-TK phosphorylation, and reducing cell viability. This makes it a promising candidate as an alternative HER2-TK inhibitor for breast cancer treatment. Since the phage library construction was performed using primers specific to human VH gene segments to amplify the immunoglobulin genes of a nonimmune camel, only the beginning and the end parts of the VH were humanized leaving room for further humanization of the anti-HER2 TK Nb.

In this study, the universal humanized Nb scaffold, named h-NbBcII10$_{FGLA}$, was chosen for anti-HER2 TK CDR loop grafting for humanization. As shown in Fig. 1, a Framework-2 mutation was performed to improve the biochemical properties of the nanobody. Nanobody hallmark amino acids (positions 42, 49, 50, and 52) play an essential role in adapting from human antibodies and compensating for the former VL side. The E49G and R50L mutations were introduced to maximize humanization by incorporating human-specific residues. These mutations had neutral effects on antigen-binding affinity but contributed to nanobody stabilization. Position 42 was retained to maintain nanobody integrity and antigen interaction. The Gly/Ala-52 residue, commonly found in camelid immunoglobulins, was introduced. Specifically, Ala-52 was chosen to preserve the hydrophobic properties of Trp-52, as observed in humans. However, mutations to bulkier amino acids at this position resulted in reduced thermal and conformational stability [3].

Codon optimization has advanced as a strategy to improve protein expression efficiency by multiple codons that can code for the same amino acid. First identified in *E. coli*, optimal codon usage has shown a strong association with increased gene expression, fueling its application from basic research to fields like biopharmaceuticals and vaccine development [11]. Therefore, the VHH genes were codon-

optimized using GenSmart™ codon optimization tool provided by Genscript, Inc. (USA).

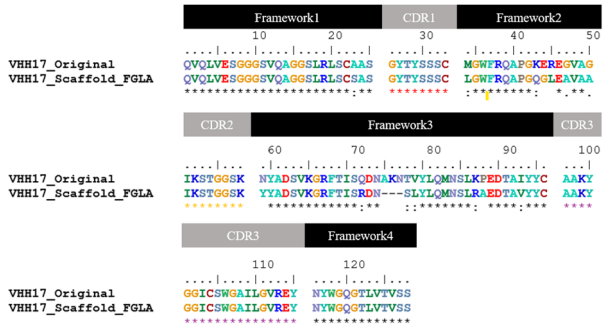

Fig. 1. Amino acid sequences of the original VHH17 compared with the humanized VHH17. (Asterisk (*): conservative amino acid; Colon (:): similar chemical property amino acid; Blank space (): non-conservative amino acid; Period (.): A semi-conservative substitution amino acid)

B. Cloning of the Original and Humanized VHH17 Nbs

The VHH genes were synthesized in the pUC19 vector by GenScript, Inc. To produce VHHs, the genes needed to be subcloned into an expression vector. In this study, pET-28(a) was chosen, as it utilizes the T7 promoter system derived from phage, which is commonly used to achieve high recombinant protein expression in *E. coli* [12]. The VHH genes were subcloned into pET28a-scFv-GCN4, which contains both FLAG and 6x-His tags at the C-terminal of the scFv. The FLAG epitope tag is highly efficient for protein detection due to its specificity, reducing non-specific protein bands during Western blot analysis.

The VHH17 gene fragments were amplified via Polymerase Chain Reaction (PCR) using primers that appended 5' NcoI and 3' SalI cut sites. The amplified fragments were then ligated into NcoI-SalI digested pET28a-scFv-GCN4. This section describes the analysis of the original VHH gene cloning. The humanized VHH gene cloning was performed similarly (data not shown).

To confirm the expected sizes of the digested PCR fragments and the vector, gel electrophoresis was performed (Fig. 2), ensuring the precise cloning of VHH17 genes for subsequent expression analysis.

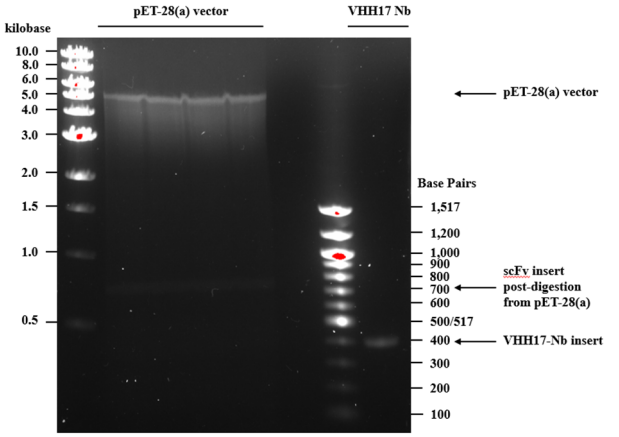

Fig. 2. Gel electrophoresis of PCR amplification of the original VHH gene and pET28a-scFv-GCN4 vector after digestion with the restriction enzymes, NcoI and SalI.

Following digestion, the observed size of the VHH17 PCR product was approximately 400 base pairs (bp), consistent with the expected size of the Nb sequence (384 bp). Similarly, the observed size of the pET-28(a) vector post-digestion was approximately 5,000 bp, aligning with the known size of the plasmid vector (5257 bp). After ligation, the mixture was transformed into electrocompetent NEB10β *E. coli* cells, chosen for their high transformation efficiency. The sequences of all constructs were verified using the Sanger sequencing method (U2Bio Co., Ltd., South Korea), as shown in Fig. 3.

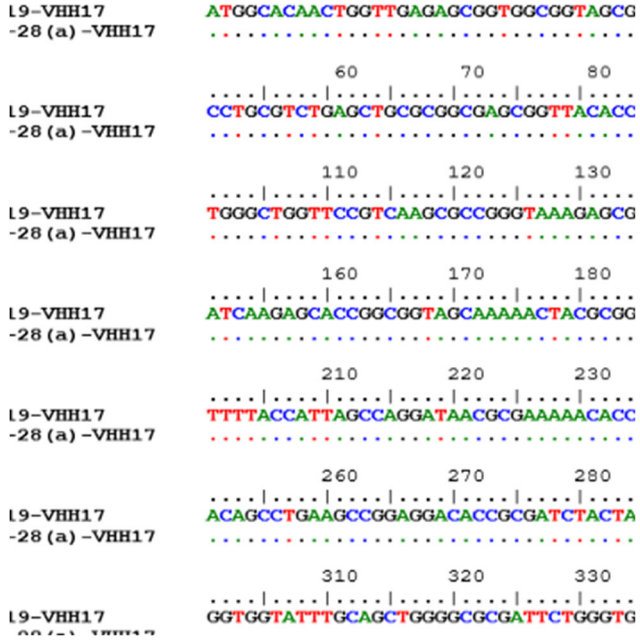

Fig. 3. The original VHH17 sequences comparison between the sequenced verified pET-28(a)-VHH17 and the chemically synthesized pUC19-VHH17.

C. Comparison of Expressions at the Reference Condition from Various E. coli Strains

A literature review of 6x-His-tagged Nb expression revealed that many studies reported an optimal expression temperature of 20 °C [7–10]. Therefore, we first investigated the expression efficiency of VHH17 in four different *E. coli* strains under the same conditions previously reported for affinity purification of a Nb selective for the native surface epitope of *Alexandrium minutum*, a small dinoflagellate responsible for algal blooms in many coastal regions worldwide. This Nb was expressed by induction with 0.5 mM IPTG for 24 hours [7].

Original VHH17 Nb expression in BL21(DE3) cells was used as the reference condition. SHuffle® T7 (SF(K12)) and SHuffle® T7 Express (SF(B)) strains are derivatives of *E. coli* strains K12 and BL21, respectively. These strains are engineered to produce proteins containing disulfide bonds in the cytoplasm and are suitable for T7 promoter-driven protein expression as they express a chromosomal copy of T7 RNA polymerase.

Fig. 4 shows that both VHH17 variants were expressed in BL21(DE3), SF(B), and SF(K12) at 20 °C with 0.5 mM IPTG after 24 hours of induction but were barely detectable in MC4100(DE3). The overall expression of the original VHH17 was higher than that of the humanized VHH17. To quantitatively analyze VHH17 expression, densitometry analysis was performed (Fig. 5). Interestingly, the original VHH17 expression in both B strains (BL21(DE3) and SF(B)) was significantly higher compared to the humanized VHH17.

Nonetheless, producing an Nb with higher amino acid

sequence similarity to the human counterpart is more desirable. Humanization reduces the risk of immunogenicity in humans, which can lead to side effects or ineffectiveness. Encouragingly, preliminary results showed that the expression of humanized VHH17 in SF(K12) was still at an acceptable level. Therefore, further optimization of production conditions for humanized VHH17 focused on its expression in SF(K12).

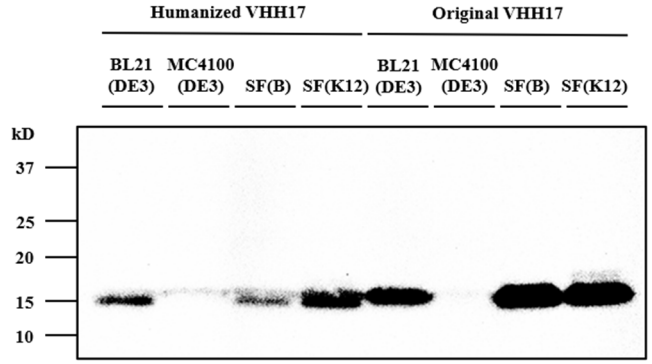

Fig. 4. Expressions of the original and humanized VHH17 from pET-28(a) in various strains of *E. coli* as indicated. The expression was conducted at 20 °C with 0.5 mM IPTG induction for 24 hours.

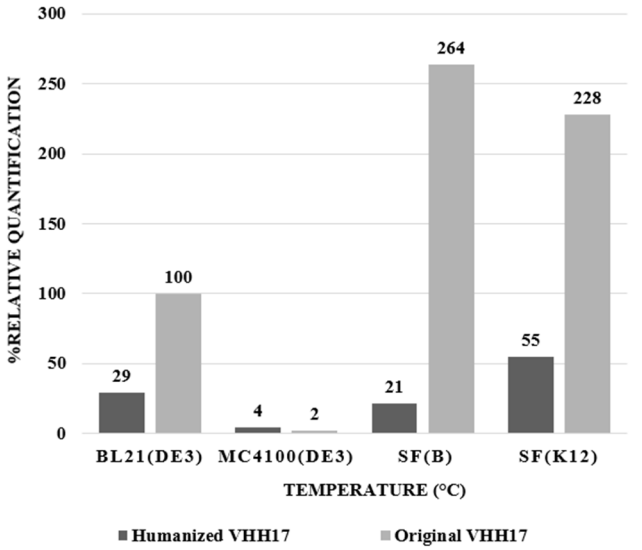

Fig. 5. Relative quantification of the original and humanized VHH17 expression conducted at 20 °C with 0.5 mM IPTG induction for 24 hours. Band intensities were quantified using Bio-Rad ChemiDoc™ MP Imaging System based on the Western blot analysis in Fig. 4.

D. Investigation of the Effects of Temperature and Induction on the VHH Production

For the production of antibody fragments, *E. coli* has been utilized for many decades. A comprehensive review covering over 250 studies has been reported [13]. At this stage, we focused on the effects of temperature and induction on humanized VHH17 production. The typical temperature range for heterologous protein expression in *E. coli* is between 16 °C to 37 °C, and IPTG concentrations used for induction in the pET vector system typically range from 0.01 mM to 1 mM.

We attempted to express humanized VHH17 in the cytoplasm of *E. coli* SF(K12) at three expression temperatures, i.e., 20 °C, 30 °C, and 37 °C and three IPTG concentrations, i.e., 0.05 mM, 0.5 mM, and 1 mM. Interestingly, humanized VHH17 was expressed well only at 20 °C (Fig. 6). Many studies have reported that lowering the growth temperature of *E. coli* below 30 °C significantly enhances the yield of recombinant antibody fragments, likely by slowing the translation rate, which promotes proper Ig-like folding and minimizes aggregation [13].

In our experience, the production of some Nbs can be higher at higher temperatures, such as 30 °C or 37 °C. Therefore, these conditions were included in this study. However, for humanized VHH17, a drastic reduction in cytoplasmic Nb production was observed as the temperature increased. When comparing the effects of IPTG induction, densitometry analysis revealed that 1 mM IPTG induction resulted in approximately 53% higher production than 0.5 mM IPTG induction, which was originally used as the reference condition (Fig. 7).

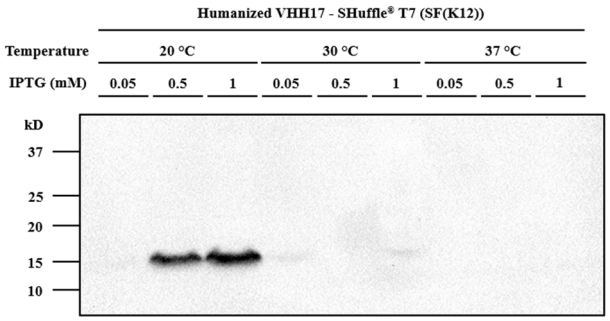

Fig. 6. Expressions of the humanized VHH17 from pET-28(a) in *E. coli* SF(K12). The expression was conducted at 20 °C, 30 °C, and 37 °C with 0.05 mM, 0.5 mM and 1 mM IPTG as indicated. Cells were collected 24 hours after induction.

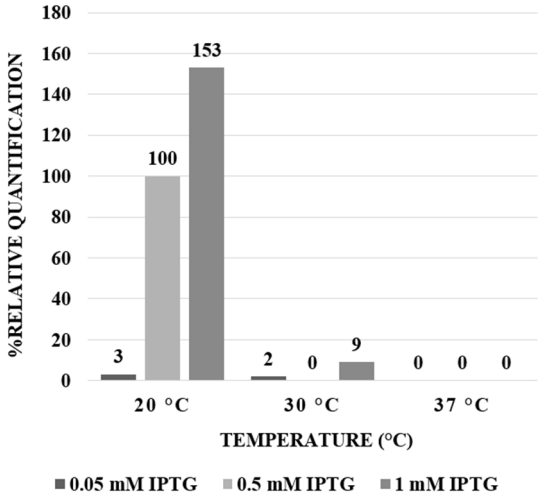

Fig. 7. Relative quantification of the humanized VHH17 expression at various conditions as indicated. Band intensities were quantified using Bio-Rad ChemiDoc™ MP Imaging System based on the Western blot analysis in Fig. 6.

IV. Conclusion

E. coli is a widely used cell factory for producing recombinant proteins. Traditionally, antibody fragments have been produced in the *E. coli* periplasm, as disulfide-bond formation is required for their proper folding. However, since the cytoplasmic space of *E. coli* is considerably larger than the periplasmic space, engineered *E. coli* strains have been developed to enhance recombinant protein production, including antibody-like fragments. Advances in methods for producing Nbs within the cytoplasm of *E. coli* have the potential to transform their industrial production. These

approaches not only reduce production costs but also facilitate the design and genetic modification of recombinant proteins, allowing *E. coli* as an efficient host system for recombinant protein production. In this study, we first grafted VHH17, a Nb against HER2-TK, onto a universal scaffold and constructed an *E. coli* codon-optimized gene within a universal humanized Nb scaffold. We then investigated the effects of *E. coli* strains, temperature, and induction on the expression of humanized VHH17 Nb. Comparative expression experiments revealed that the optimal conditions for humanized VHH17 production were achieved using SF(K12) at 20 °C with 1 mM IPTG following 24 hours of induction. Notably, higher induction resulted in a 53% increase in protein expression relative to the baseline. Despite these findings, the expression efficiency of humanized VHH17 remains lower than that of the original VHH17, indicating the necessity for further large-scale pilot studies to increase yield. Additionally, it is crucial to assess the specificity and binding affinity of the newly grafted VHH17 to HER2-TK for improved analytical results.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

MR, NS, WK, and KI conducted the research. MR, LT, TL, and DW-Z analyzed the data; MR, LT, KC, and DW-Z validated the data and performed formal analysis; MR, LT, and DW-Z prepared the original draft; MR, LT, KC, and DW-Z reviewed and edited the manuscript; DW-Z supervised the research and acquired funding; all authors had approved the final version.

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