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# Establishment of a signal peptide with cross-species compatibility for functional antibody expression in both *Escherichia coli* and Chinese hamster ovary cells

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ABSTRACT

Signal peptides are short peptides located at the N-terminus of secreted proteins. They characteristically have three domains; a basic region at the N-terminus (n-region), a central hydrophobic core (h-region) and a carboxy-terminal cleavage region (c-region). Although hundreds of different signal peptides have been identified, it has not been completely understood how their features enable signal peptides to influence protein expression. Antibody-derived signal peptides are often used to prepare recombinant antibodies expressed by eukaryotic cells, especially Chinese hamster ovary (CHO) cells. However, when prokaryotic *Escherichia coli* (*E. coli*) are utilized in drug discovery processes, such as for phage display selection or antibody humanization, signal peptides have been selected separately due to the differences in the expression systems between the species. In this study, we successfully established a signal peptide that enables a functional antibody to be expressed in both prokaryotic and eukaryotic cells by focusing on the importance of having an Ala residue in the c-region of the signal sequence. We found that changing Ser to Ala at only two positions significantly augmented the anti-HER2 antigen binding fragment (Fab) expression in *E. coli*. In addition, this altered signal peptide also retained the ability to express functional anti-HER2 antibody in CHO cells. Taken together, the present findings indicate that the signal peptide can promote functional antibody expression in both prokaryotic *E. coli* and eukaryotic CHO cells. This finding will contribute to the understanding of signal peptides and accelerate therapeutic antibody research.


## 1. Introduction

Antibodies are major therapeutic proteins used for cancer, allergies and other unmet medical needs. Fabs are the functional antibody domains used for antigen binding, and are emerging therapeutic proteins [1]. The protein expression efficiency is a key area of research and development that need to be improved for the generation of therapeutic antibodies and Fabs. Both prokaryotic and eukaryotic cells have been utilized in the various drug discovery processes such as phage display selection [2] or humanization [3] to synthesize the lead monoclonal antibody candidate. *Escherichia coli* has been a dominant prokaryote host used for economical manufacturing processes [4], and it has been successfully used to produce clinically available proteins such as insulin [5], growth hormone [6], G-CSF (granulocyte colony-stimulating factor) and some other therapeutic proteins [7]. On the other hand, CHO cells have been used as eukaryotic cells to generate recombinant proteins which need post transcription modifications for the therapeutic activities, such as EPO (Erythropoetin) [8] and numerous monoclonal antibodies [9].

The N-terminus of synthesized secretory proteins contains a peptide sequence consisting of 15–30 amino acids referred to as a signal peptide [10]. Signal peptides control the entry of virtually all proteins into the secretory pathway, in both eukaryotes and prokaryotes [11]. The domain of signal peptides from various proteins are commonly described as a positively charged n-region, followed by a hydrophobic h-region and a neutral but polar c-region [12]. These are cleaved off after the proteins are translocated through cellular membrane. The cleavage sites usually conform to the established rule for residues at positions −3 and −1 from the cleavage site in prokaryotes. The rule states that small and neutral residues (Gly, Ser, Ala) at positions −3 and −1 may enhance the rate of the cleavage process by bacterial signal peptidase I (Lep) [13–15]. Comparative studies of signal peptides cleaved by signal peptidases I and II suggested that these are different for the c-region. A well-conserved signal sequence, Leu-Ala-(Gly,Ala) followed by Cys, defines the bacterial signal peptidase II (Lsp)

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cleavage site [16]. In terms of length, signal peptides from eukaryotes tend to have slightly shorter n-, h-, and c-regions than signal peptides from Gram-negative bacteria. Conversely, Gram-negative bacterial signal peptides have shorter regions than Gram-positive bacteria [17]. The structural homology among those signal peptides is such that they can similarly work when they are transferred to another species, but usually result in a lower level of secretion.

Although hundreds of different signal peptides have been identified and compared structurally, most of the previous studies on the function of signal peptides have been limited to bacteria and yeast. Signal peptide modification of human IL-2 was investigated on correlation between the sequence properties and rate of endostatin or alkaline phosphatase protein secretion in a human malignant cell line, MDA-MB-435 [18]. This study revealed that basicity and hydrophobicity at h-region were key features in human signal peptides. Cross-species compatibility has been demonstrated separately in examples such as rice α-amylase expressed in *Saccharomyces cerevisiae* [19], human CD23 expressed in a baculovirus system with a signal sequence derived from a gram-positive bacterium, Staphylococcal protein A [20]. However, the compatibility of mammalian signal peptides with bacterial hosts remains unknown, which leads to poor cleavage rates and low yields of secreted proteins, especially antibodies. On the other hand, bacterial expression systems have been utilized during the drug discovery process and the findings of studies performed to develop these systems also support an industrial manufacture process using *E. coli* or CHO as a host cell [21].

To this end, we tested a range of *E. coli* and mammalian signal peptides on the Fab expression in *E. coli* or the antibody expression in CHO cells, but these signal peptides did not show compatible expression at all. Amino acid residues at positions −3 and −1 in the c-region were previously reported to be important for the recognition and cleavage of signal peptidase I in *E. coli* [22]. In particular, the residue at position −1 must be a small side chain such as Gly, Ser or Ala to allow for efficient protein secretion. In this study, we tested a signal peptide derived from a mouse antibody because this signal peptide has three continuous Ser residues at the C-terminal. Even though this signal sequence was fused to the N-terminus of Fab, we failed to express Fab in *E. coli*. Next, we precisely examined an amino acid residue substitution from Ser to Ala at both positions −3 and −1 in the c-region and found that Fab expression could be significantly enhanced by this modification in *E. coli*. We also found that this altered signal sequence retained the ability to induce the expression of the functional antibody in CHO cells. These data clearly indicated that we succeeded in creating a compatible signal peptide to express a functional antibody in both prokaryotic and eukaryotic cells.

## 2. Materials and methods

### 2.1. Cloning and expression of recombinant proteins

The genes encoding a humanized anti-HER2 Fab (4D5) [23] were synthesized for insertion into an expression vector after codon optimization for each host, including signal sequences. For bacterial expression, the variable regions, including the signal peptides of anti-HER2 Fab were separately subcloned into a pFLAG-based vector (Sigma Aldrich) to prepare the promoter and a series of signal peptide derivatives. Plasmid vectors were introduced into *E. coli* strain W3110 by the heat-shock method. For eukaryotic cell expression, human anti-HER2 antibody expression plasmids were generated by cloning them into the pKANTEX93 vector [24]. These plasmid vectors were introduced into the DG44 CHO cell line by electroporation and G418-resistant clones were obtained. The supernatants were applied to 1 mL of Mabselect resin (GE Healthcare) and purified to prepare anti-HER2 antibodies. The soluble human HER2 extracellular domain fused to a His-tag was generated by polymerase chain reaction (PCR) and cloned into the pKANTEX93 vector. This vector was introduced into rat hybridoma cell line YB2/0 by an electroporation method and G418-resistant clones were thus obtained. The supernatant was added to 0.5 mL of TALON resin (Clontech) and soluble human HER2 antigen was purified.

### 2.2. Fermentation of E. coli expressing Fab fragment

Vector-transformed cultures of W3110 were incubated at 37 °C in Luria–Bertani broth. The pre-culture fluids were inoculated into 200 mL of Super Broth to start fermentation in a 1 L Erlenmeyer flask [25]. When the absorbance at 600 nm reached 2.0, the temperature was shifted to 20 °C and the bacterial culture continued overnight after the addition of 0.1 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG). After centrifugation of the culture broth, cell pellets were lysed using the B-PER extraction reagent (Thermo Scientific), the cell lysates were clarified by centrifugation (4500*g*, 20 min). After filtration (0.22-μm), the supernatant was applied to TALON resin (Clontech) and Fab fragments were purified.

### 2.3. Detection and quantification of secreted Fab protein

The yields of each Fab sample in the culture fluid were measured by sandwich ELISA. Flat-bottomed 96-well plates were first coated with 100 μl of 1:1000 diluted anti-human IgG F(ab)′2 fragment (Jackson Immuno Research) in carbonate-bicarbonate buffer. After being blocked with Super blocking buffer in TBS (Thermo Scientific), purified Fabs were incubated on the plates at room temperature for 1 h. After being washed with PBS containing 0.05% Tween 20 (wash buffer), the binding Fabs were detected using HRP-labeled goat anti-human kappa antibody (Southern Biotech) with the substrate tetramethylbenzidine (Wako). The reaction was stopped with the addition of 0.5 mmol/L sulfuric acid (Wako), and the absorbance at 450 nm/595 nm was measured by a Sunrise plate reader (Tecan). The total amount of Fab was calculated by a standard curve using reference samples.

### 2.4. Polyacrylamide gel electrophoresis (SDS–PAGE)

A total of 10 μl of purified samples were mixed with 2 μl of 6× sample buffer (Nacalai) and loaded onto Mini-PROTEAN TGX gels (Bio-rad) in running buffer. A 10 μl aliquot of SpectraTM Multicolor Broad Range Protein ladder marker (Thermo Scientific) was loaded onto the gel simultaneously. The running conditions followed a standard protocol (300 V, 25 min). To visualize the proteins after electrophoresis, the gels were stained with Coomassie Blue for 1 h and then were destained for 1 h. The destained gels were photographed using a LAS-3000 system (FUJI FILM).

### 2.5. Antigen binding assay

The antigen binding properties of the purified antibody or Fab were measured by ELISA. Flat-bottomed 96-well plates were coated with human HER2 antigen in TBS. After being blocked with Super blocking buffer in TBS (Thermo Scientific), the purified antibodies or Fabs were quantified and the same amount of each antibody or Fab was added to the plate. The plate was incubated at room temperature for 1 h. After being washed with TBS containing 0.05% Tween 20, bound Fabs or antibodies were detected using an HRP-labeled goat anti-human light chain antibody (Bethyl Laboratories) or an HRP-labeled goat anti-human IgG antibody (American Qualex) with the substrate tetramethylbenzidine (Wako). The