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ARTICLE

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Tailoring translational strength using Kozak sequence variants improves bispecific antibody assembly and reduces product-related impurities in CHO cells

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Genentech

Abstract

Optimal production of bispecific antibodies (bsAb) requires efficient and tailored co-expression and assembly of two distinct heavy and two distinct light chains. Here, we describe a novel technology to modulate the translational strength of antibody chains via Kozak sequence variants to produce bsAb in a single cell line. In this study, we designed and screened a large Kozak sequence library to identify 10 independent variants that can modulate protein expression levels from approximately 0.2 to 1.3-fold compared with the wild-type sequence in transient transfection. We used a combination of several of these variants, covering a wide range of translational strength, to develop stable single cell Chinese hamster ovary bispecific cell lines and compared the results with those obtained from the wild-type sequence. A significant increase in bispecific antibody assembly with a concomitant reduction in the level of product-related impurities was observed. Our findings suggest that for production of bsAb it can be advantageous to modify translational strength for selected protein chains to improve overall yield and product quality. By extension, tuning of translational strength can also be applied to improving the production of a wide variety of heterologous proteins.

KEYWORDS

bispecific antibody production, chain ratios, Kozak sequence, mammalian cell culture, translational strength

1 | INTRODUCTION

Therapeutic antibodies represent the most successful class of biological drugs. In the past 30 years, 50+ therapeutic antibodies have been approved for clinical use in various indications, including cancer, autoimmune, infectious, and vascular diseases (Ayyar, Arora, & O'Kennedy, 2016). Given the remarkable impact, therapeutic antibodies represent one of the fastest growing areas of the pharmaceutical industry.

Due to the fact that most diseases involve several parallel signaling pathways, simultaneous inhibition and/or activation of receptors and ligands may improve therapeutic efficacy. Therefore, bispecific antibodies (bsAb) or complex antibody formats, which have the ability to bind to multiple epitopes, show promise for application in multifaceted disease therapies, such as inflammatory and autoimmune diseases (Chan & Carter, 2010), cancer (Dong et al., 2011; Kou et al., 2010) and infectious diseases (De Bernardis et al., 2007; Laventie et al., 2011) among others (Brinkmann & Kontermann, 2017; Carter & Lazar, 2018; Kontermann, 2012). During the past decade, over 60 different bsAb formats have been developed to modulate pharmacokinetic and distribution properties (Spiess, Zhai, & Carter, 2015). However, the production process for

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bsAb has been challenging due to promiscuous pairing of different antibody heavy chains and light chains (HCs and LCs). With random pairing up to 10 different combinations are possible, with only one being the properly assembled bsAb. This problem limits the production of bsAb in sufficient quantity and quality to support preclinical and clinical development needs.

Antibody framework engineering has been used to significantly alleviate the chain mispairing problem to promote heterodimerization of the HC or LC with knobs-into-holes and crossMab technologies, respectively (Atwell, Ridgway, Wells, & Carter, 1997; Klein, Schaefer, & Regula, 2016; Merchant et al., 1998; Ridgway, Presta, & Carter, 1996). Despite these gains, framework designs alone cannot completely resolve the problem and extensive optimization of the antibody production process is still required. One manufacturing process solution is to employ separate expression of half-antibodies followed by in vitro assembly to produce bsAb (Spiess et al., 2013). However, this approach is time-consuming and costly, requiring the generation of two distinct stable cell lines, as well as separate yet coordinated manufacturing campaigns. Therefore, generation of bsAb in a single cell line would be an attractive option, with the caveat that this approach requires more extensive early optimization to obtain a cell line capable of producing the highest levels of properly assembled bsAb with minimal product-related impurities.

Chinese hamster ovary (CHO) cells are currently the primary host for the biopharmaceutical industry (Dhara, Naik, Majewska, & Betenbaugh, 2018; Kunert & Reinhart, 2016; Tripathi & Shrivastava, 2019; Walsh, 2018; Wurm, 2004). Compared with other mammalian cells, CHO cells combine the advantages of robust cell growth and effective posttranslational modification with the well-established standards of good manufacturing practices (Kunert & Reinhart, 2016). Bioprocessing innovations and cell engineering efforts have improved product titer (Ayyar, Arora, & Ravi, 2017; Kelley, Kiss, & Laird, 2018); however, uncharacterized cellular processes and gene regulatory mechanisms can still hinder cell growth, specific productivity, and protein quality (Fischer, Handrick, & Otte, 2015; Harcum & Lee, 2016; Kuo et al., 2017).

Translational efficiency is a systematic approach that may be used to control recombinant protein expression in mammalian cells. Polypeptide synthesis is initiated at the translation initiation site (TIS), a region consisting of the start codon and its adjacent bases. In eukaryotes, translation initiation typically follows the scanning mechanism model (Hinnebusch, 2014; Kozak, 1978). This model postulates that the ribosomal preinitiation complex, comprising the small 40S ribosomal subunit, Met-tRNA, and several initiation factors, binds at the 5′ end of messenger RNA (mRNA) and advances linearly in the 3′ direction in search of a start codon. After positioning the small 40S ribosomal subunit at the start codon, the initiation factors dissociate, the large subunit binds to the small 40S ribosomal subunit forming the ribosomal complex and translation starts (Nanda, Saini, Munoz, Hinnebusch, & Lorsch, 2013; Pestova & Kolupaeva, 2002). However, initiation is not always restricted to the start codon (ATG) nearest the 5′ end. If the first ATG codon occurs in an optimal context, the ribosomal complex typically initiates translation, but if the TIS around the first ATG triplet is suboptimal, some 40S subunits may bypass that site and initiate further downstream (Chappell, Edelman, & Mauro, 2006; Hinnebusch, 2017). Therefore, cells can control protein translation levels by tuning the TIS such that the sequence around the start codon plays a major role in the initiation and subsequent enhancement of translation efficiency (Ivanov, Loughran, Sachs, & Atkins, 2010; Kozak, 1991a, 1991b; Sonenberg & Hinnebusch, 2009).

Kozak has reported CCRCCATGG (R = A or G; start codon underlined) to be a highly efficient mammalian TIS (Kozak, 1981; Noderer et al., 2014). Within that sequence, the purine in position −3 (three nucleotides upstream of the ATG codon) is most highly conserved in vertebrate messenger RNA (Hernandez, Osnaya, & Perez-Martinez, 2019; Kozak, 1987a). Point mutational studies provide evidence of the importance of A or G in position −3, and G in position +4 (immediately following the ATG codon) as crucial for optimal translational efficiency (Kozak, 1997). The Kozak consensus sequence differs in length and nucleotide composition between species but is conserved for most genes within the species (Cavener, 1987; Grzegorski, Chiari, Robbins, Kish, & Kahana, 2014; Gupta, Rangan, Ramesh, & Gupta, 2016; Hamilton, Watanabe, & de Boer, 1987; Kozak, 1984, 1987a, 1987b). Not surprisingly, point mutations in the Kozak sequence affect translation initiation both in higher (Kozak, 1986) and lower (Dvir et al., 2013; Wallace et al., 2020) eukaryotes and have been associated with the development of human diseases, including cancer, and metabolic disorders (Mohan et al., 2014; Sonenberg & Hinnebusch, 2009).

In this study, we generated a Kozak sequence library and indirectly determined the translational strength of each variant by measurement of titer in transient transfection. Screening of the library resulted in 10 Kozak sequence variants encompassing a wide range of strengths from 0.2 to 1.3 relative to the WT Kozak. Next, we used a mix of the WT Kozak plus four representative Kozak sequence variants covering the translational range to generate a single cell bsAb antibody stable cell line. Our study revealed that tailoring translational strength for the production of bsAb subunits by varying the Kozak sequence generated a higher percentage of correctly assembled bsAb and reduced the presence of undesired species.

2 | MATERIALS AND METHODS

2.1 | Cell line and cell culture

A cell line derived from the CHO-K1 host, property of Genentech, was used for transient transfection assays and for stable cell line development via a proprietary targeted integration site. CHO cell seed trains were cultured in a proprietary Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F12)-based medium in 125 ml shake flask vessels shaking at 150 rpm, 37°C and 5% CO_2. Cells were passaged at a seeding density of 3×10^5 cells/ml every 3–4 days.

2.2 | Kozak sequence variants constructions

DNA of the Kozak sequence variants of the Fc-fusion Protein A or B was synthesized by Genewiz, Inc. Variants were cloned into a transient transfection vector containing an ampicillin resistance marker and where the gene of interest is under the transcriptional control of the CMV promoter. Sequence verification was done using standard methods.

2.3 | DNA library construction

Fc-fusion Protein B was used as the gene of interest for the synthetic DNA Kozak library sequence. Positions −5, −4, −3, −2, −1, and +4 relative to the start codon were diversified to any of the four nucleotides. The library was synthesized by Genewiz, Inc. The library was cloned into a transient transfection vector containing an ampicillin resistance marker and where the gene of interest is under the transcriptional control of the CMV promoter. The library was transfected into Max Efficiency® DH5α competent cells (Invitrogen, Carlsbad, CA) using several dilutions to optimize the selection of individual colonies. DNA prep of individual colonies was done according to the manufacturer's recommendation (QIAGEN, Hilden, Germany). Sequencing was performed using standard methods.

As shown in Figure S1, nucleotide distribution for the screened variants was random and diverse per position, indicating that the library screening was not biased. The criteria to select variants round to round were reproducibility between transient transfections, and between different DNA preparations, and preservation of the nucleotide diversity.

2.4 | Transient transfection

CHO cells were transfected with transient transfection plasmids using lipofectamine 2000 CD according to the manufacturer's recommendation (Invitrogen). As internal controls, DNA of established well-expressing and poorly expressing antibodies were also completed side-by-side with the test cases. Briefly, DNA vector (2 μg) plus 10 μl of lipofectamine in 500 μl of media were incubated for 30 min at room temperature. The transfection complex was transferred to a 96-well plate (FalconR) containing exponentially growing CHO cells in a proprietary DMEM/F12- based medium plus 5% dalyzed fetal bovine serum for a total working volume of approximately 2.5 ml. Transfected cells were cultured at 37°C, 5% CO_2, and 80% humidity. Cell culture medium was exchanged at 24 hr posttransfection for production medium. Cells were then cultivated at 33°C for approximately an additional 24 hr. Each transfection was performed in biological duplicates. For antibody titer determination, supernatant samples were collected at 48 hr posttransfection and assayed in replicate by homogeneous time-resolved FRET (HTRF) assay.

2.5 | Stable vector constructions

Two targeted integration antibody expression vectors were used to develop stable cell lines for the bispecific antibody. The expression vectors contain two separate cytomegalovirus (CMV) promoters to direct the transcription of both the HC and LC as two separate units. The *Streptomyces alboniger* puromycin-N-acetyltransferase (Vara, Portela, Ortin, & Jimenez, 1986) gene was used as the selection marker in one plasmid and a gene for a fusion protein containing the positive-negative selection marker HyTK that confers resistance to hygromycin was used as the selectable marker in the other plasmid. Generation of the 25 expression vectors per arm was done by cloning a combination of five HCs by five LC under different Kozak sequence variants to generate the Kozak mix pools. Two expression plasmids, one for each antibody with the heavy and LCs under WT Kozak sequence, were used to develop the WT Kozak pools.

2.6 | Stable cell line development

CHO cells were transfected using MaxCyte STX Transfection System according to the manufacturer's recommendation (MaxCyte, Gaithersburg, MD). Transfected cells were pooled into two separate pools and selected with selective medium containing puromycin 5 μg/ml and 1-(2'-deoxy-2'-fluoro-1-beta-D-arabinofuranosyl-5-iodo) uracil 0.5 μM. After recovery, one pool was subjected to single cell cloning (SCC) by limiting dilution at 1 cell/well into 384-well clear, flat-bottom, tissue culture treated plates (Corning Inc., Corning, NY) using Wellmate Microplate dispenser (Thermo Matrix) with an Integra Viafill sterile 8 channel tubing of 0.5 mm pore size (IntegrΛ). Plates were incubated at 37°C, 5% CO_2. Three to 4 weeks after seeding, 704 individual colonies were picked into 96-well plates (Corning Inc.) and after 2 days were evaluated for antibody production using HTRF. The top 48 and subsequently the top 24 antibody-expressing clones were assayed via HTRF, and the top 11 single cell clones were adapted to shake flask growth and evaluated in a fed-batch production assay. An additional seven clones that showed medium and low harvested cell culture fluid (HCCF) titers compared with the top clones were also evaluated side-by-side with the top clones.

2.7 | Shake flask fed-batch production assay

Fed-batch production cultures were performed in shake flasks (Corning Inc.) with proprietary chemically defined basal medium along with bolus proprietary feeds on Days 7 and 10. Cells were seeded at 1.0×10^6 cells/ml. A temperature shift from 37°C to 35°C was carried out on Day 3. Day 14 titers were determined using Protein A affinity chromatography with UV detection. Percent viability and viable cell count was determined on Days 0, 3, 7, 10, and 14 using Vi-Cell XR instrument (Beckman Coulter). Glucose and lactate concentrations were monitored on Days 7 and 14 using a Bioprofile 400 Analyzer (Nova Biomedical).

2.8 | Product quality analysis

Standard product quality analyses were conducted by subjecting the total Protein-A purified antibody to nonreducing capillary electrophoresis sodium dodecyl sulfate. A TECAN Evo200 was used to automate the sample preparation. All samples were diluted to 1 mg/ml to ensure consistent liquid handling and maintain optimal dye-to-protein ratios. Prepared samples were immediately analyzed by the labChip GXII and the data processed using Chromeleon software. Signal intensity, peak profiles and relative peak area distributions were assessed. A half antibody and full antibody are define them as 75 and 150 kDa species.

2.9 | Mass spectrometry

To identify and quantify protein product-related variants, qualitative analysis and mass determination was performed on an Agilent 6230 Time-of-Flight mass spectrometer using HPLC-Chip separation and ion source. Data deconvolution was performed using MassHunter software. We determined ion abundances for the bispecific as well as for species with incorrect LC and HC stoichiometry.

2.10 | Calculation

When calculating the composition of the sample, we orthogonally weighted liquid-chromatography mass spectrometry (LC-MS) and capillary electrophoresis sodium dodecyl sulfate (CE-SDS) results to account for the fact that full-length species, with two HC and two LC, are expected to ionize with different efficiency than half-antibodies with one HC and one LC. We multiplied the ion abundance for each full-length species by the CE-SDS measured percent main peak:

$$\%Bispecific = \frac{(Abundanceofbispecificby LC-MS)(\%main-peakbyCE-SDS)}{(Sumofallfull-lengthspeciesabundances)}$$

A similar calculation was performed for each species, so that the CE-SDS percent main peak was split among each species reported, weighted by their abundances measured on LC-MS.

2.11 | Antibody surface staining protocol

For antibody surface staining, about 2 million cells were pelleted and washed twice with phosphate-buffered saline (PBS) buffer. Cells were then re-suspended in 0.5 ml of PBS containing anti-human immunoglobulin G (IgG; H + L) allophycocyanin-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) at a 1:100 dilution and incubated for 20 min at 37°C with shaking. The unstained control sample was re-suspended in PBS without antibody. Empty host was also stained to set up the gates. After 20 min of incubation with the staining antibody, cells were washed once with PBS and re-suspended in 400 µl of PBS. Then, cells were analyzed in a FACscan (Attune NxT Flow Cytometer, Life Technologies). For each analysis 20,000 events were recorded.

2.12 | Genomic DNA extraction, polymerase chain reaction amplification, and sequencing analysis

Genomic DNA extraction was performed using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer's manual. DNA was quantified with a SimpliNano Microvolume Spectrophotometer (GE Lifesciences). Polymerase chain reaction (PCR) amplification was performed using Q5® Hot Start High-Fidelity 2X Master Mix (New England Biolabs) with 1 µg of gDNA used as a template. An annealing temperature of 64°C and 30 cycles were used. Specific annealing primers in the variable region of each chain were designed to distinguish the chains between molecules. The following forward (F) and reverse (R) primers were used to amplify Ab1 HC, Ab1 LC, Ab2 HC, and Ab2 LC: HC-Ab1: F-5′-GATACCAGCACCAGCACCGCCT-3′ and R-5′-ATGGGCGGTAGGCGTGTACGG-3′; LC-Ab1: F-5′-CTGAACAG CCGCACCCGCAA-3′ and R-5′-ATGGGCGGTAGGCGTGTACGG-3′; HC-Ab2: 5′-GTGATTTGGCGCGGCGGCA-3′ and R-5′-ATGGGCGG TAGGCGTGTACGG-3′; and LC-Ab2: 5′-GTGCGCAACCTGGTGGT GTGG-3′ and R-5′-ATGGGCGGTAGGCGTGTACGG-3′. PCR products were cleaned with PCR purification Kit (QIAGEN) following manufacturer's instructions and the correct size was analyzed by 12-well 2% premade agarose gels (Thermo Fisher Scientific). Sequencing was performed using the next primers: SEQ1: 5′-AACGGTGCA TTGGAACGCGG-3′ and SEQ2: 5′-TGGCTTCGTTAGAACGCAGC-3′. The Kozak sequence variant for each chain in each clone was confirmed at least twice. For some clones sequencing data could not be obtained. These were Kozak Mix clones 11and 21L for Ab2-HC and Ab1-LC, respectively (Figure 5c and Figure S4d).

2.13 | Gene copy number determination

DNA copy number was carried out as previously described (Carver et al., 2020).

2.14 | Quantitative real-time PCR analysis

RNA samples were purified using QIAGEN RNeasy Mini Kit (cat# 74014) according to the manufacturer's instructions. To determine the mRNA level of HC and LC, TaqMan quantitative real-time PCR (qRT-PCR) assay was performed using TaqMan® RNA-to-Ct™ 1-Step Kit (cat# 4392653; Thermo Fisher Scientific). Each TaqMan RT-PCR reaction contained 1X RT-PCR mix, 1X RT enzyme mix, 900 nM forward primer, 900 nM reverse primer, 250 µM probe, and 2 ng/µl purified RNA sample. The thermal cycling conditions were 15 min at 48°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were processed on the

QuantStudio™ 6 Flex Real-Time PCR System (Life Technologies). Data were analyzed using Design and Analysis Software Version 2.3 (Life Technologies) after TaqMan RT-PCR amplification. The relative expression levels of HC and LC were determined based on Delta Ct analysis method. The expression level of reference gene, GAPDH, was used to normalize different RNA samples in each reaction. The primers used in this study were designed using primer express v3.0 (Life Technologies). Primers and probe sequences used were as following:

HC-1 Forward primer: TCA AGG ACT ACT TCC CCG AAC C. HC-1 Reverse primer: TAG AGT CCT GAG GAC TGT AGG ACA GC. Probe: VIC-ACG GTG TCG TGG AAC TCA GGC GC-TAMRA.
LC-1 Forward primer: GCT GCA CCA TCT GTC TTC ATC T. LC-1 Reverse primer: GCA CAC AAC AGA AGC AGT TCC A. Probe: VIC-CCC GCC ATC TGA TGA GCA GTT GAA-TAMRA.
HC-2 Forward primer: TCAAGGACTACTTCCCCGAACC. HC-2 Reverse primer: TAGAGTCCTGAGGACTGTAGGACAGC. Probe: FAM-ACGGTGTCGTGGAACTCAGGCGC-TAMRA.
LC-2 Forward primer: TGACGCTGAGCAAAGCAGAC. LC-2 Reverse primer: CAGGCCCTGATGGGTGAC. Probe: FAM-ACGAGAAACACAAAGTCTACGCCTGCGA-TAMRA.
GAPDH Forward primer: GGGAAGGCCATCACCATCTT. GAPDH Reverse Primer: ATACTCGGCGCCAGCATC. Probe: FAM-CAGGAGCGAGATCCCGCCAACAT-TAMRA.

3 | RESULTS

3.1 | Identifying Kozak sequence variants to modulate protein translation in CHO cells

To confirm that Kozak sequence variants can modulate expression in CHO cells, presumably through translation, we tested four different previously described (Kozak, 1987a) Kozak sequences with nucleotide variations in selected positions upstream of the start codon of an Fc-fusion protein. These variants are depicted in Figure 1a and were expected to lower the translational strength of the Kozak sequence. Transient transfection of two different Fc-fusion proteins (A and B) bearing these Kozak variants showed a reduction in Fc-fusion protein titer in CHO cells (Figure 1b). As anticipated, the strongest and weakest Kozak sequence variants evaluated (CCGCCATGG and CCTTTATGG, respectively), had similar or roughly half of the translational strength as that of the consensus (WT) Kozak sequence based on Fc-fusion protein titer (Figure 1b).

Next we designed a library of Kozak sequences using Fc-fusion Protein B to increase the range of available translational strengths. The library randomized the five bases upstream (positions −5, −4, −3, −2, and −1), and one base downstream (position +4), of the start codon to any of the four DNA bases (Figure 2a). The expected diversity of this library was around 4100 Kozak sequence variants. Randomization at position +4 created an amino acid change in the 2nd position of the signal sequence from glycine to arginine when this position was occupied by an adenine (A) or cytosine (C).

The library screening process is depicted in Figure 2b. Following transformation into competent *E. coli* cells, approximately 100 randomly selected individual variants were sequenced to verify the diversity of the library (data not shown). Once the diversity of the library was confirmed, 111 correct variants, those with the correct coding sequence, as well as controls, WT Kozak and WT Kozak with a C or A at the position +4 to control the change in the second amino acid of the signal sequence, were then screened using transient transfection. Fc-fusion protein titers, measured at 48 hr post-transfection, showed a broad range of expression levels (Figure 2c). The results encompassed a nearly continuous range of translational strength from 0.05 to 1.54 units of normalized titer, where 1.0 corresponds to expression with the WT Kozak sequence. These Kozak

(a)

Name	Sequence
Kz.WT / consensus	CCACC ATG G
Kz.G (A_{-3}> G)	CCGCC ATG G
Kz.T (A_{-3}>T)	CCTCC ATG G
Kz.TAG (ACC_{-3}>TAG)	CCTAG ATG G
Kz.3T (ACC_{-3}>TTT)	CCTTT ATG G

(b)

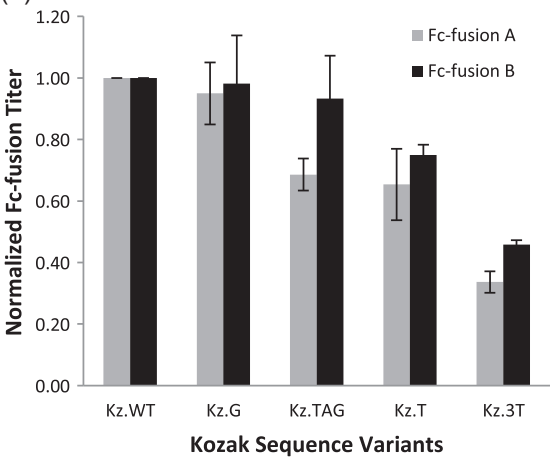
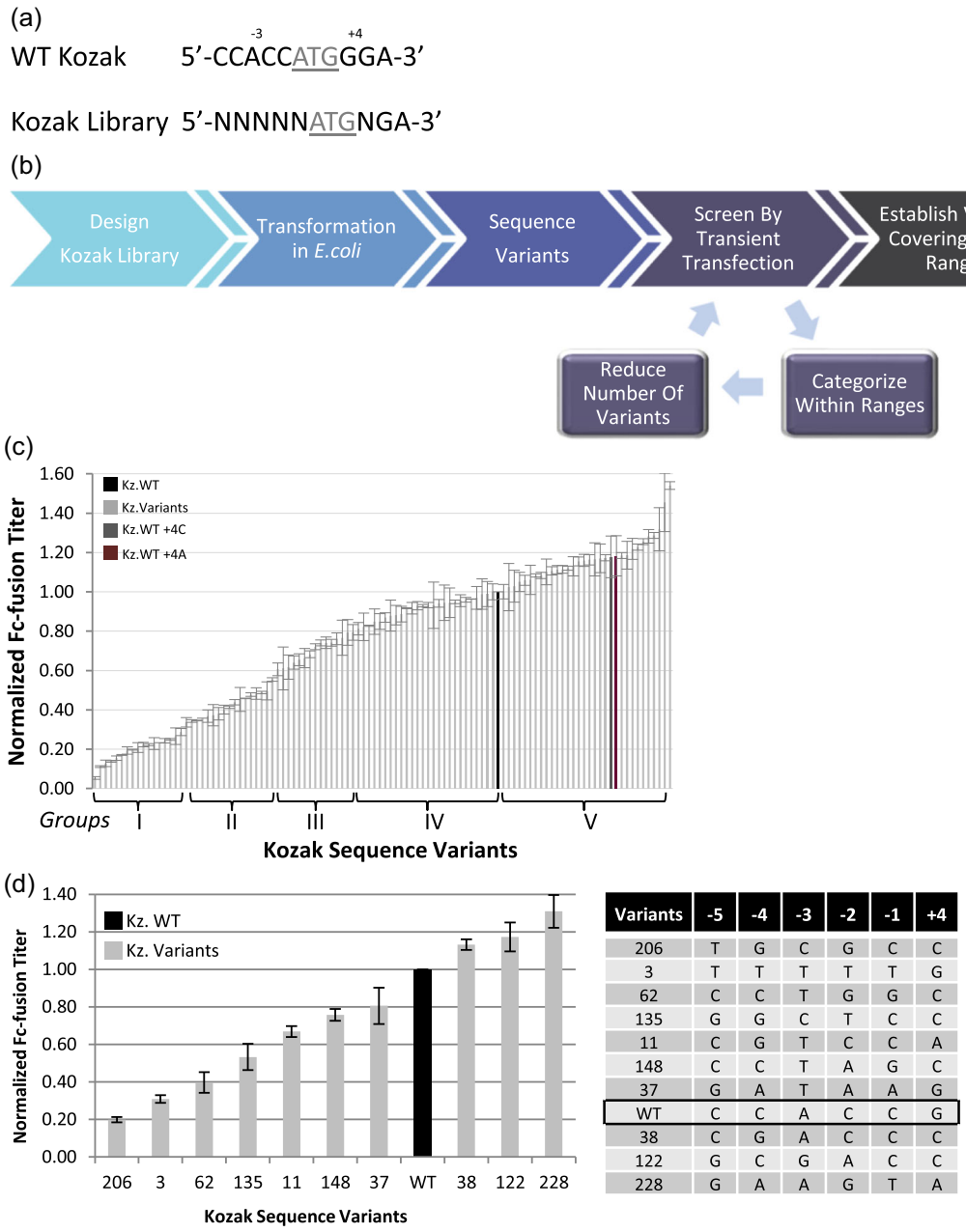


FIGURE 1 Proof of concept that using Kozak sequence variants can modulate expression levels. (a) Name and nucleotide sequence of the Kozak sequence variants designed and analyzed by transient transfection. The start codon is gray and underlined. (b) Normalized transient transfection Fc-fusion titer for the different Kozak sequence variants. Data is presented as mean values and error bars represent the standard deviation (*SD*) of four independent transfections. For each transfection, a new DNA prep was prepared to mitigate any DNA prep artifacts. Kz, Kozak. WT, Wild-type



Variants	-5	-4	-3	-2	-1	+4
206	T	G	C	G	C	C
3	T	T	T	T	T	G
62	C	C	T	G	G	C
135	G	G	C	T	C	C
11	C	G	T	C	C	A
148	C	C	T	A	G	C
37	G	A	T	A	A	G
WT	C	C	A	C	C	G
38	C	G	A	C	C	C
122	G	C	G	A	C	C
228	G	A	A	G	T	A

FIGURE 2 Designing and screening a Kozak sequence library by transient transfection. (a) Sequence of WT Kozak and the Kozak Library. The start codon is gray and underlined. Positions −3 and +4 are indicated. N represents any of the four nucleotides either A, T, G, or C. (b) Illustration of the process used to identify Kozak variants covering a range of translational levels. (c) Normalized transient transfection Fc-fusion titer for the 111 Kozak sequence variants. Data is presented as mean values and error bars represent the *SD* of two independent transfections. For each transfection, a new DNA prep was prepared to mitigate any DNA prep artifacts. Except for the preference of guanosine and thymine in most of the positions for the weakest group, and arginine as second amino acid of the signal sequence for the strongest group, other obvious correlations between antibody production and Kozak sequence variant were not apparent (data not shown). (d) Normalized transient transfection Fc-fusion titer for selected Kozak sequence variants. Data is presented as mean values and error bars represent the *SD* of 11 independent transfections. The table depicts the name and nucleotide sequence of the Kozak sequence variants. WT Kozak is framed with a box. Kz, Kozak. *SD*, standard deviation; WT, Wild-type [Color figure can be viewed at wileyonlinelibrary.com]

variants provided a wide range of translational strength with which to potentially modulate protein expression of different bsAb chains. Regardless of the sequence upstream of the start codon, Kozak variants with higher translational strength than the WT Kozak had the presence of A or C in the +4 position in common (Figure 2c).

We next classified the Kozak variants into five groups (as indicated on the x-axis of Figure 2c) based on titer. These groups had translational strengths corresponding to normalized titer of: Group I 0.05–0.3; Group II 0.31–0.6; Group III 0.61–0.8; Group IV 0.81–1.00 and Group V 1.01–1.34. Several rounds of transient transfections

were performed to assess consistency and select 10 representative Kozak variants with translational strengths covering normalized titer ranges of 0.2–1.3, compared with the WT, at 0.1-unit expression intervals (Figure 2d).

3.2 | Generation of bispecific stable clones under a mix of Kozak sequence variants

The application of Kozak sequence variants as a technology to modulate protein expression in stable CHO host cell lines was then evaluated. Since identification of a unique parental antibody ratio to merge into a bispecific format is needed for high productivity bsAb (Ho et al., 2013), the capability of Kozak sequence variants to yield higher assembly and production of bsAb in a single cell line was tested. A representative panel of four variants plus the WT Kozak was selected for the evaluation. The relative protein production strengths of these variants were approximately 0.3, 0.5, 0.75, 1.0, and 1.3-fold of the WT (Kozak Sequence Variants #3, #135, #148, WT and #228, respectively; Figure 2d). This approach covered the broad range of expression levels observed in transient transfections.

The bispecific antibody Ab1/Ab2 was selected as a model for this study on the basis of the following criteria: First, a well-characterized process development platform was available for Ab1/Ab2 to facilitate the analysis and characterization of the product. Second, Ab1 is a poorly expressing molecule with bottleneck downstream of transcription, while Ab2 represents an example of a well-expressed molecule (data not shown). Thus, this is an appropriate scenario to examine the capability of Kozak sequence variants to create successful chain pairings from a challenging situation. Third, Ab1/Ab2 has shown high effectiveness as anticancer agent in primates, creating interest in process development optimization for this molecule.

To preferentially promote HC heterodimerization, the HCs of both Ab1 and Ab2 carried hole and knob mutations, respectively, and charge steering mutations were used to mitigate LC mispairings (Dillon et al., 2017). A combination of HC and LC, each with the five selected Kozak sequence variants, was used to develop 25 expression vectors per arm (one arm = Ab1 HC + Ab1 LC). A mix of the 50 plasmids was transfected into CHO cells (referred to as Kozak Mix). The diversity generated with this approach reached up to a potential 625 different chain ratio combinations. As a reference value, the four chains of bsAb cloned using the WT Kozak sequence were also transfected into CHO cells and carried in parallel (referred to as WT Kozak). Two different transfections were performed for both conditions (Kozak Mix and WT Kozak). Transfection efficiency was monitored by cell surface staining for total IgG and measured by FACS analysis (Figure S2a). Similar transfection efficiency was observed for both Kozak Mix and WT Kozak pools. Following the transfection of expression plasmids, stable cell pools were established by antibiotic selection (Figure S2b). After recovery, one stable pool from each transfection for each condition was selected and subjected to SCC. Plates were analyzed for clones and 704 clones per pool and condition were picked for analysis. Primary screening based on HCCF titer showed differences in the profile and the titer between conditions (Figure 3). As expected, Kozak Mix clones showed lower titer and a more pronounced decrease in the titer than WT Kozak clones. This result matches the planned diversity of translational strength combinations in Kozak Mix clones while the profile of WT Kozak clones was as expected for clones carrying the same translational strength combination. Several rounds of screening based on HCCF titer were performed as part of scale up to determine the 11 top clones per pool per transfection and for each condition, which resulted in 22 clones in total for each condition. Due to the fact that each Kozak Mix clone has the potential to carry a different Kozak sequence variant combination and to facilitate understanding of the differences between combinations, an additional seven Kozak Mix clones that showed medium and low HCCF titers compared with the top clones were also selected for further analysis.

3.3 | Shake-flask performance of bispecific stable clones derived from a mix of Kozak sequence variants

A 14-day shake flask fed-batch production was used to evaluate the productivity and bispecific assembly of each individual clone. All investigated clones showed comparable viabilities

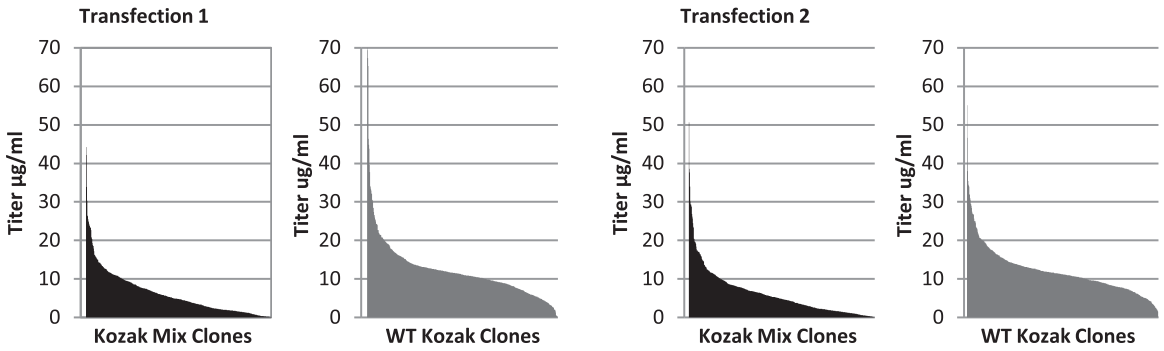


FIGURE 3 Bispecific stable cell line development process in a single cell line. Homogeneous time resolved fluorescence (HTRF) titer of 704 Kozak Mix clones and WT Kozak clones for each transfection. Data is framed in pairs based on transfection number. Transfection number is indicated. WT, wild-type

across the production assay (Figure 3a). Final viabilities at Day 14 were approximately 80–95% in both conditions except for one Kozak Mix clone with a final viability of 74%. Similarly, viable cell counts showed an exponential growth rate until Day 7 followed by a plateau phase for all individual clones from Days 7–14 (Figure S3b). For each clone, general titer (includes all species, such as correctly/incorrectly assembled bsAb, all half antibody versions and unwanted side products), product quality and assembly efficiency were measured at Day 14. As shown in Figure 4a, individual WT Kozak clones showed higher general antibody titer than individual Kozak Mix clones in both transfections.

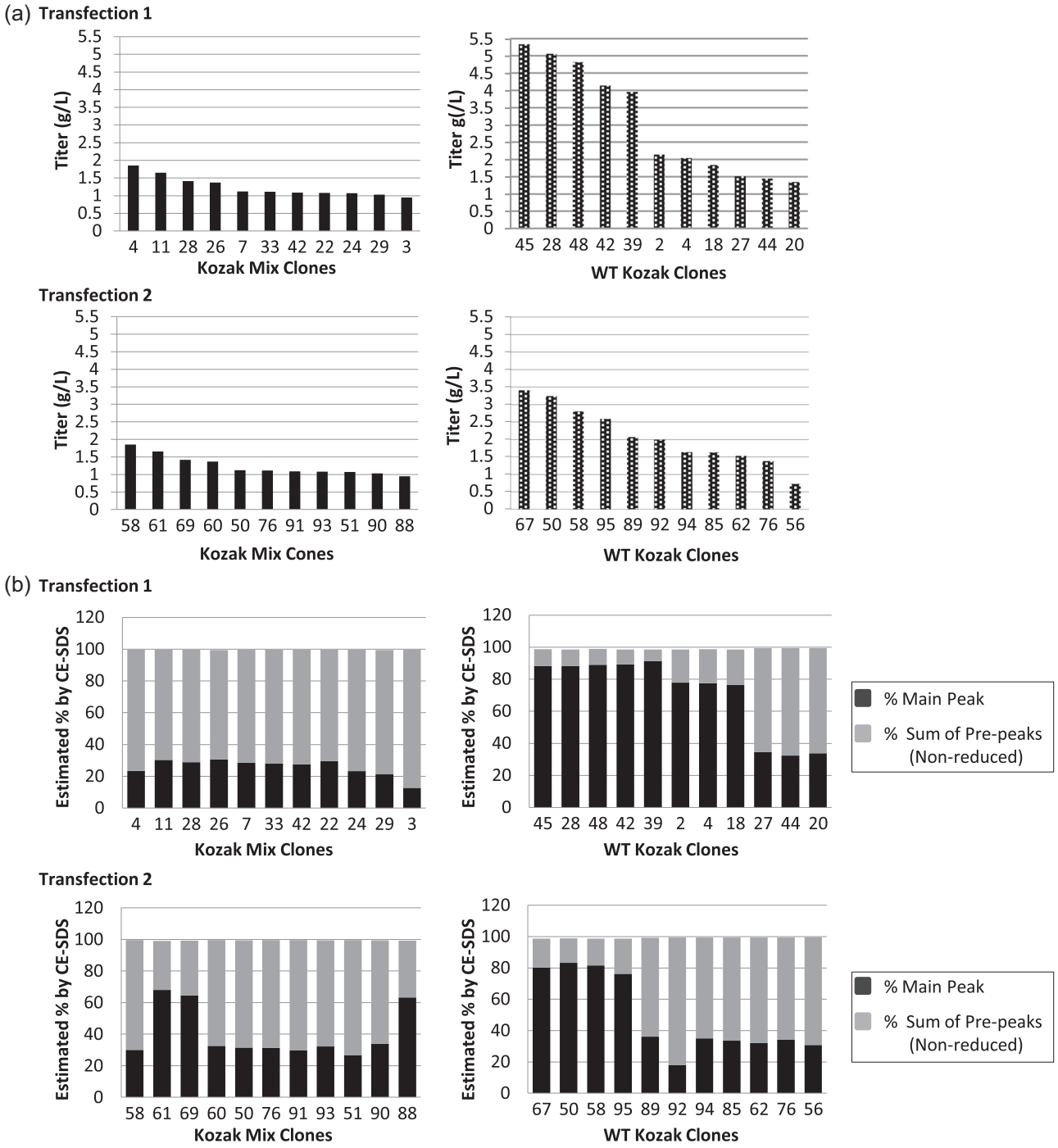


FIGURE 4 General titer and product quality of the top clones derived from each pool. (a) General titer for the top 22 of Kozak Mix clones and the top 22 WT Kozak clones (11 per transfection). Titer was measured at Day 14 of a shake flask fed-batch production. (b) Nonreducing capillary electrophoresis sodium dodecyl sulfate (CE-SDS) for the top 22 Kozak Mix clones and the top 22 WT Kozak clones (11 per transfection). Transfection number is indicated. WT, wild-type

Product quality was initially assessed using nonreducing CE-SDS to provide information about molecular weight forms and other impurities. This technique enabled quantification of two formats: The main peak that equates with the full antibody (full-ab), including both correctly and incorrectly assembled bsAb; and, the sum of pre-peaks comprising low-molecular weight species such as half-antibodies. Among the top 22 clones there were more WT Kozak clones with a higher percentage of full-ab (Figure 4b). This result was consistent with and expected from the experimental design. In the case of WT Kozak, a total of 1408 clones were screened for only 1 construct. However, for the Kozak Mix condition, 1,408 clones were screened for a total of 625 constructs, which favored finding a higher number of WT Kozak clones with high product quality. The percentage of full-ab varied among clone. Three Kozak Mix clones from transfection 2 ranged around 60% full-ab. The percentage full-ab for the Kozak Mix clones then dropped to approximately 30%. The Kozak Mix clones from Transfection 1 showed a main peak of around 30% (Figure 4b). This result is in agreement with the translational strength diversity found in the Kozak Mix pool. In addition, not all the highest antibody-producing clones (general titer) were linked with the highest content of full-ab. In general, the best WT Kozak clones ranged between 80% and 90% full-ab production. Similar to Kozak Mix clones, WT Kozak clones showed variability in the percentage of main peak depending on the clone. Also, a correlation between high general titer and high content of main peak in these clones was observed (Figure 4b).

In parallel, the seven additional Kozak Mix clones with medium and low HCCF titer relative to the top clones were evaluated. General titer of these clones from the fed-batch experiment showed a ranking similar to that observed in the primary screening (Figure S4a). Product quality by the CE-SDS varied across clones as shown (Figure S4b). Again, there was no direct correlation between general titer and the highest content of full-ab. Notably, several Kozak Mix clones with a lower general titer than the top 22 clones showed similar percentage of full-ab at approximately 50–60% (Figure S4b).

3.4 | Kozak sequence variants enhanced bispecific assembly in stable clones

The top four clones from CE-SDS analysis per condition and transfection were analyzed to identify and quantitate incorrect pairing of HC and LC. IgG was recovered from cell culture medium using Protein A affinity chromatography, and the resulting pools were analyzed by intact LC-MS. Following data deconvolution, several product-related variants including half-antibodies (HC + LC), homodimers (two of the same half-antibody) and mispaired LC-HC species were identified. Using a calculation reported in the methods section, the LC-MS data was used to orthogonally weight the CE-SDS results to report percentages of species that otherwise overlap analytically on CE-SDS due to having similar mass.

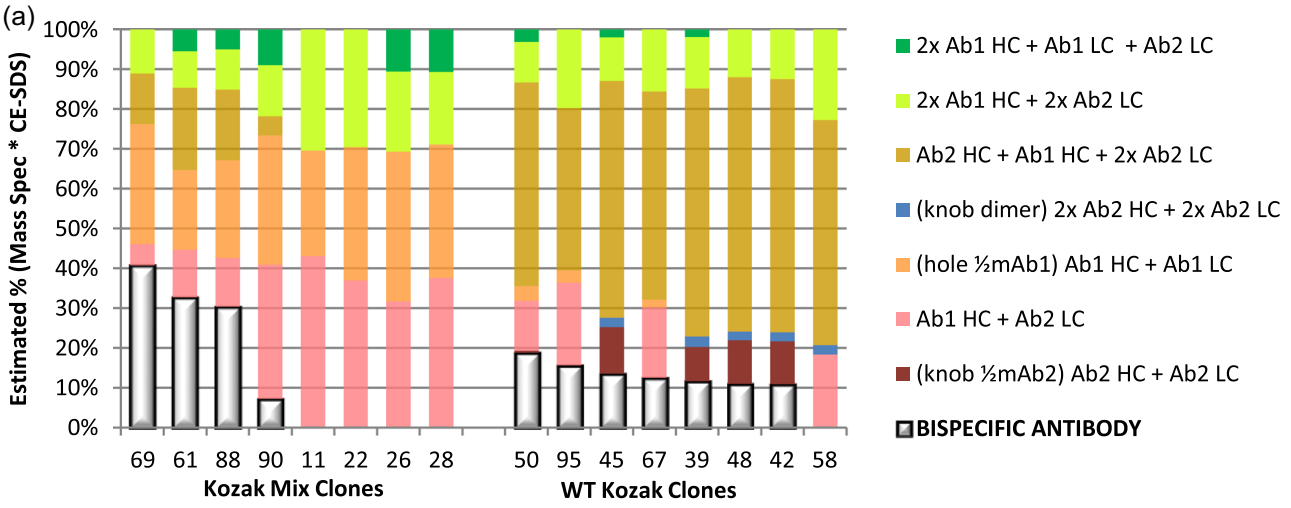
Seven different full-length species and three half-antibody species were identified and their relative abundances compared. The top Kozak Mix clone showed 40% correctly assembled bsAb, which is more than double that of the top WT Kozak clone (~18%; Figure 5a). Since every Kozak Mix clone potentially carried a different Kozak variant combination, each clone showed a different percentage of bsAb assembly, whereas WT Kozak clones which carried the same combination exhibited a much narrower range of percent bsAb assembly (Figure 5a). Remarkably, the incorrectly assembled bispecific format Ab2 HC + Ab1 HC + 2x Ab2 LC, which had a mispairing of the LC, ranged from 41% to 64% of the full-ab produced by the WT Kozak clones (Figure 5a). These data point out the importance of tuning LC translational levels to achieve correct bsAb assembly when using single cell line production. Analysis by mass spectrometry of Kozak Mix clones with the lowest full-ab formation yield (Kozak Mix Clone 90, 11, 22, 26, and 28), as measured by CE-SDS, confirmed that the majority of the general titer produced by those clones was half-ab. The formats were the Ab1 HC + Ab2 LC and (hole ½ mAb1) Ab1 HC + Ab1 LC. The bsAb species produced by these clones were mostly the incorrectly assembled 2x Ab1 HC + 2x Ab2 LC and also the bsAb species 2x Ab1 HC + Ab1 LC + Ab2 LC see above in a lower percentage (Figure 5a).

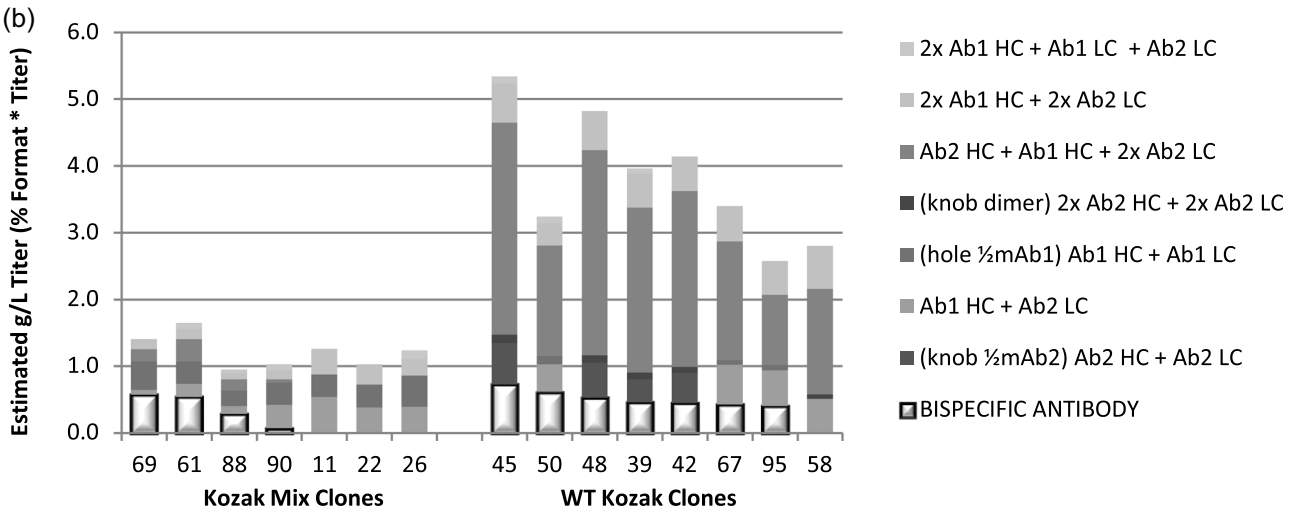
Once the percentage of each species was determined, the effective bispecific titer was calculated (Figure 5b). Because some Kozak Mix clones had higher bsAb assembly than WT Kozak clones, Kozak Mix clones overcame the deficit in general titer indicated previously (Figure 4a). The effective bsAb titer was similar between the top clone per condition: 0.7 and 0.6 g/L for the top WT Kozak and Kozak Mix clone, respectively.

Importantly, Kozak Mix clones also presented fewer product-related impurities than the WT Kozak clones (Figure 5b). Purification of the correctly assembled bsAb from a large number of almost identical potential byproducts is challenging. As some of the byproducts display close similarity to the target bsAb, their removal downstream often comes at the expense of yield or product quality. New analytical methods are frequently needed to differentiate between the product of interest and these unique impurities, and may require high-cost manufacturing and complicated technologies (Kroner & Hubbuch, 2013). Kozak Mix clones minimized the impurities and product-related variant formation (Figures 5b and 6a) demonstrating the benefit of this approach to produce bsAb in a single cell line.

3.5 | Selectively combining weaker Kozak variants with stronger variants results in increased bsAb assembly efficiency and effective titer

The heterogeneity of bsAb assembly efficiency and product quality observed in the Kozak Mix clones suggested that the clones expressed the chains under different Kozak sequence variants. to



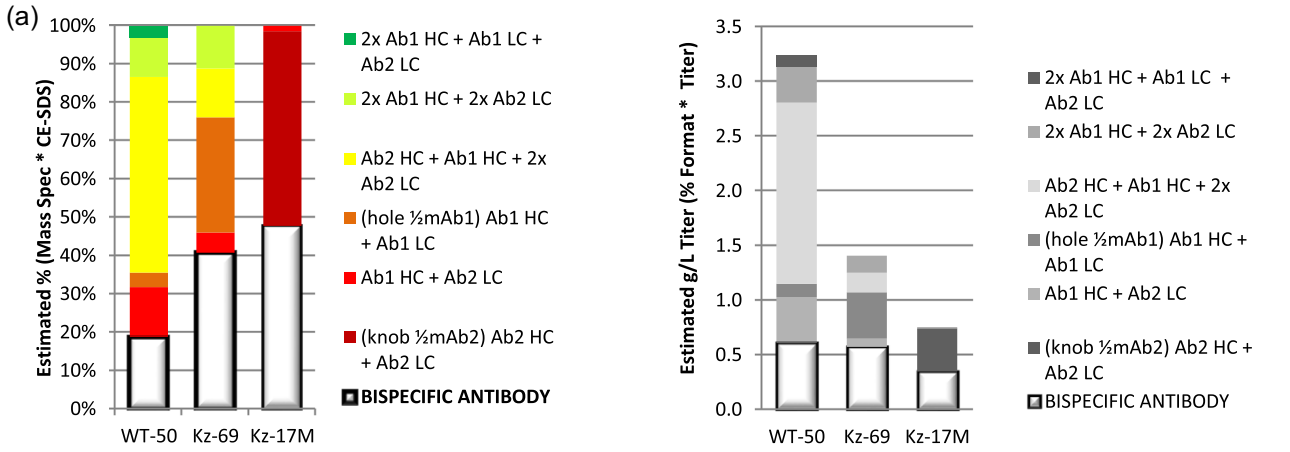


(c)

	Kozak Sequence Variant			
	Ab1		Ab2	
Kozak Mix Clone	HC	LC	HC	LC
69	*Kz.228*	*Kz.228*	*Kz.228*	*WT*
61	*Kz.228*	*WT*	*Kz.228*	*Kz.228*
88	*Kz.228*	*WT*	*Kz.228*	*Kz.228*
90	*Kz.228*	*WT*	*Kz.148*	*WT*
11	*Kz.228*	*WT*		*Kz.228*
22	*WT*	*Kz.228*	*Kz.135*	*WT*
26	*Kz.228*	*Kz.228*	*Kz.135*	*Kz.228*
28	*WT*	*WT*	*Kz.3*	*WT*

Kozak Variant	Translational Strength
Kz.228	1.3
WT	1
Kz.148	0.75
Kz.135	0.5
Kz.3	0.3

FIGURE 5 Mass spectrometry data, effective titer, and Kozak sequence variant combinations of bispecific stable clones. (a) Quantification of bispecific antibody and half-antibody species by mass spectrometry for eight Kozak Mix clones and eight WT Kozak clones. Each species is indicated in the legend. Correctly assembled bispecific antibody is highlighted in white. (b) Effective titer of each bispecific and half-antibody species calculated as percentage of each species multiplied by general titer. Correctly assembled bispecific antibody effective titer is highlighted in white. (c) Kozak sequence variant combinations for the Kozak Mix clones. Highlighted in gray the sequencing data that could not be obtained. The name and translational strength of each Kozak sequence variant is shown on the right. HC, heavy chain; LC, light chain; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]



(b)

Kozak Mix Clone	Titer (g/L)	CE-SDS: Main Peak (%)	CE-SDS: Pre-Peaks (%)	MS: LMWS (1HC+1LC): (knob ½mAb2) Ab2 HC + Ab2 LC	MS: LMWS (1HC+1LC): Ab1 HC + Ab2 LC	MS: Main Peak (2LC+ 2 HC): BISPECIFIC ANTIBODY	Kozak Sequence Variant: Ab1 HC	Kozak Sequence Variant: Ab1 LC	Kozak Sequence Variant: Ab2 HC	Kozak Sequence Variant: Ab2 LC
17M	0.8	47.8	52.2	50.8%	1.4%	47.8%	*Kz.135*	*Kz.135*	*WT*	*Kz.228*

Kozak Variant	Translational Strength
Kz.228	1.3
WT	1
Kz. 135	0.5

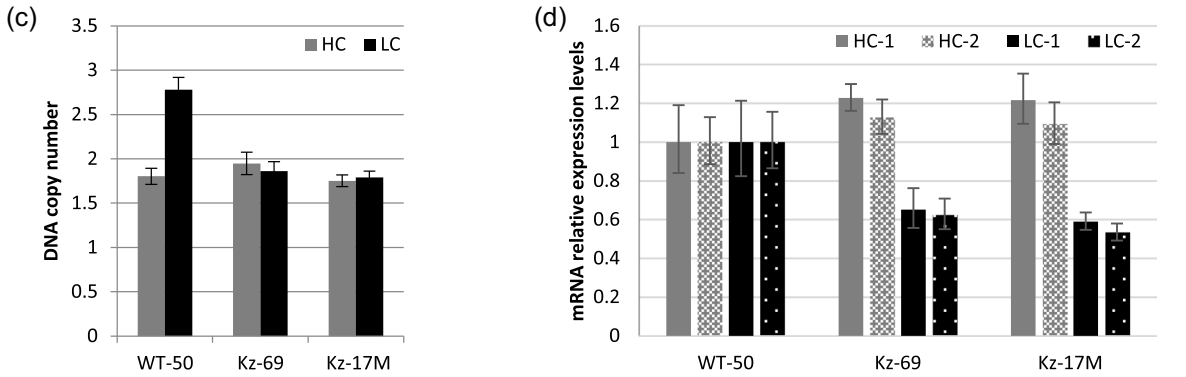


FIGURE 6 Mass spectrometry data, effective titer, and Kozak sequence variant combinations for Kozak Mix Clone 17 M. (a) Quantification of bispecific antibody and half-antibody species by mass spectrometry for WT Kozak Clone 50 and Kozak Mix Clones 69 and 17 M. Correctly assembled bispecific antibody is highlighted in white. The effective titer of each bispecific and half-antibody species, calculated as a percentage of each species multiplied by general titer, is shown on the right. Correctly assembled bispecific antibody effective titer is highlighted in white. (b) General titer, CE-SDS, mass spectrometry and Kozak sequence variant combination for Kozak Mix Clone 17 M. The name and translational strength of each Kozak sequence variant is shown. (c) Heavy and LC gene DNA copy number for WT Kozak Clone 50 and Kozak Mix Clones 69 and 17 M. Error bars represent the *SD* of three technical replicates. (d) mRNA expression for WT Kozak Clone 50, Kozak Mix Clones 69 and 17 M. mRNA expression for heavy and LCs were normalized to WT Kozak Clone 50. Error bars represent the *SD* of three technical replicates. CE-SDS, capillary electrophoresis sodium dodecyl sulfate; HC, heavy chain; LC, light chain; mRNA, messenger RNA; *SD*, standard deviation; WT, wild-type [Color figure can be viewed at wileyonlinelibrary.com]

identify the Kozak combinations for each clone and determine the best combination, the Kozak Mix clones were sequenced.

At one productivity extreme, Kozak Mix clones with no detectable correctly assembled bsAb (Kozak Mix Clones 90, 11, 22, 26, and 28) had the expression of Ab2 HC under the weaker Kozak sequence variants, Kz.148, Kz.135, or Kz.3 (Figure 5c). As a result, an insufficient amount of Ab2 HC was likely generated and the final product was preferentially half-ab in the format of Ab1 HC + Ab2 LC and (hole ½ mAb1) Ab1 HC + Ab1 LC (Figure 5a). Consistent with the literature, this data suggests the expression of HC can only be reduced so much before compromising full-ab assembly (Schlatter et al., 2005).

On the other hand, when a combination of both WT Kozak and the strongest Kozak sequence variant (Kz.228; relative strength of 1.3) modulated the expression of the four chains, Kozak Mix clones displayed high bsAb assembly and effective titer (Figure 5c; Kozak Mix Clones 69, 61, and 88). Interestingly, a slight LC ratio of 1.3-fold for the LC of Ab1 versus 1.0-fold for the

LC of Ab2 or vice versa exhibited by Kozak Mix Clone 69 and Kozak Mix Clone 61, respectively, significantly reduced the Ab2 LC mispairing observed in WT Kozak clones (Figure 5a; Ab2 HC + Ab1 HC + 2x Ab2 LC). These data suggest that a reduction in translation of a selected chain, relative to the translational level of the other chains, can increase bsAb assembly efficiency and improve overall product quality by limiting the accumulation of sub-species.

The striking result from Kozak Mix Clone 17 M is consistent with this conclusion. Clone 17 M was one of the additional clones taken forward, even though the primary HCCF titer was modest. Although this clone had only roughly half the effective titer of the top Kozak clones, it exhibited the highest bispecific assembly at approximately 48% (Figure 6a). MS data indicated that 100% of the full-ab corresponded with only the correct bispecific format and the half-ab species was limited almost exclusively to knob ½ mAb2 (Figure 6a,b). These data can be explained from the sequencing results. Both the heavy and LCs of Ab1 were under a weaker Kozak variant (Kz.135) and, as noted earlier, Ab1 is a difficult to express molecule. Down-regulation of both chains, as in the case of using Kz.135 with a relative strength of approximately 0.5, could alleviate bottlenecks downstream of translation, such as in protein folding, resulting in a positive impact on assembly efficiency. With respect to Ab2, HC and LC were under stronger Kozak sequences, WT Kozak and the strongest Kozak variant (Kz.228), respectively (Figure 6b). This combination, in conjunction with weaker Kozak sequences for Ab1, would result in an accumulation of Ab2 half-ab while limiting any significant buildup of other species.

To discern whether transcription was an important factor in the expression and assembly differences observed between WT Kozak Clone 50, Kozak Mix Clone 69 and Kozak Mix Clone 17 M, DNA copy number, and mRNA expression levels were quantified (Figures 6c,d). HC copy number and mRNA levels were similar between the three clones. However, WT Kozak Clone 50 contained approximately one additional copy of LC compared with Kozak Mix Clones 69 and 17 M (Figure 6c) and higher WT LC mRNA expression levels were observed accordingly (Figure 6d). These results establish a correlation between DNA copy number and transcript levels in the clones, as expected given the use of a targeted integration site for this work (Carver et al., 2020; Tadauchi et al., 2019). Remarkably, despite a very similar HC and LC mRNA level between Kozak Mix Clone 69 and 17 M, the clones show distinct protein expression profiles (Figure 6a), suggesting that the differences may be driven through a translational mechanism.

Sequencing data was also obtained for several additional clones with medium and low primary HCCF titer (Kozak Mix Clones 28 M, 21 L, 15, and 18 L). In general, most chains were expressed by weaker Kozak variants in these clones and correct bsAb assembly, when detectable, was below 10% (Figure 4c,d).

In addition to sequencing Kozak Mix clones, WT Kozak clones (WT Kozak Clones 50, 95, 45, and 39) were also sequenced. As expected, sequencing confirmed the presence of the WT Kozak sequence in the WT Kozak clones (data not shown).

4 | DISCUSSION

Although advances have been made in the engineering of complex antibody formats, such as bispecific antibodies, manufacturing these molecules in a single mammalian expression system remains challenging. Low titer and insufficient product quality are two factors that contribute to making single cell line production of bsAb difficult. As a consequence, there is a need to identify the limiting steps in the production system. Bispecific assembly efficiency, and potentially the assembly of other multi-chain formats, in a single CHO cell is constrained by the lack of control over individual chain levels. Since proportional amounts of correctly folded LCs and HCs are required for efficient bsAb assembly (Schlatter et al., 2005), designing vectors that can tailor the expression level of the individual chains in a multi-chain format could provide an advantageous tool for improving assembly efficiency, as previously demonstrated in *E. coli* with the translational initiation region variants (Simmons & Yansura, 1996; Simmons et al., 2002).

In this study, we generated a panel of CHO vectors covering a broad range of expression level by randomizing the −5, −4, −3, −2, −1, and +4 nucleotide positions. Selected vectors were then used to develop stable bsAb cell lines to assess titer, assembly efficiency, and product quality relative to control. The results show that by tuning the HC and LC expression levels with Kozak sequence variants, bispecific assembly can be increased by greater than two fold over control. Importantly, a significant reduction was observed in product-related impurities over control, potentially decreasing burden on downstream processing (Figure 5a,b). As formation of product-related variants is affected by LC and HC expression stoichiometries, Kozak sequence variants can be used to favor formation of half-antibodies which we have previously found to be easier to clear (Giese, Williams, Rodriguez, & Persson, 2018), and to decrease formation of LC mispairs which are more challenging to remove (data not shown).

Although none of the Kozak variant clones analyzed in production reached higher effective bispecific titer than control, this was not an unexpected result due to the design of the study. To screen a large number of Kozak variants to identify promising candidates and combinations under the constraint of labor-intensive stable cell line development methods, variants were mixed and selected from a single transfection. This approach severely limited the number of clones screened per variant relative to the control. Thus, this procedure, used as a first step for identifying Kozak variants, was significantly inferior to a normal cell line development process. Performing a limited number of standard stable cell line developments with only the more optimal Kozak variant combinations could likely result in clones with higher correctly assembled bsAb titer.

Interestingly, the highest percentage of correctly assembled bispecific, approximately 48%, was found in a Kozak variant clone, 17 M, with only modest titer. This clone modulates the chain expression of one arm, Ab1, a difficult to express molecule, with a weaker Kozak sequence variant. This result suggests that limiting expression can successfully alleviate bottlenecks downstream of

transcription, ultimately leading to increased productivity. Improvements in the high-throughput initial screening platform to specifically distinguish and quantify the correct bispecific format from other species, such as RapidFire High-Throughput Mass Spectrometry assay, will increase the frequency of identifying such clones with favorable assembly properties and add to the understanding of the steps limiting bsAb production.

Whether the Kozak sequences identified in this study that resulted in an improved outcome for Ab1/Ab2 can be directly applied to improve the production of another bsAb remains in question. A similar outcome could be predicted for other bsAbs, if those antibodies have similar issues to those described for Ab1/Ab2. However, the optimal sequences for any given bsAb may need to be empirically determined, as the resulting optimal antibody chain ratio likely depends on the specific expression challenges of that particular bsAb. This study identified representative Kozak sequences with defined strengths and described a cell line development workflow that can be used to apply a limited empirical approach for additional bispecific products.

The specific mechanism responsible for the differences in titer, bsAb assembly efficiency and improved product quality observed in this study will require additional experimentation, but the DNA copy number and mRNA expression level results are consistent with translation as the key component. The scanning mechanism model proposed by Kozak for translation initiation (Kozak, 1978) and the Kozak-dependent translational rate changes recently described by Acevedo, Hoermann, Schlimbach, and Teleman (2018) support this conclusion. However, at this point, it is uncertain whether other mechanisms may also be playing a role. For example, the library randomization included the +4 position, possibly leading to a change in the second amino acid of the signal sequence from glycine to a positively charged amino acid, arginine. For variants with this change, the presence of a positively charged amino acid at the N-terminus of the signal sequence may be improving protein translocation, as noted previously in both mammalian systems and *E.coli* (Clerico, Maki, & Gierasch, 2008; Gennity, Goldstein, & Inouye, 1990; Guo et al., 2018). Nonetheless, independent of the precise mechanism, the technique described has demonstrated usefulness in the production of bispecific antibodies and could be applicable for resolving a wide variety of protein expression problems.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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