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Expression of antibodies using single open reading frame (sORF) vector design

Demonstration of manufacturing feasibility

Wendy R. Gion,* Rachel A. Davis-Taber, Dean A. Regier, Emma Fung, Limary Medina, Ling C. Santora, Sahana Bose, Alexander V. Ivanov, Barbara A. Perilli-Palmer, Chris M. Chumsae, Joseph G. Matuck, Yune Z. Kunes† and Gerald R. Carson

AbbVie; Worcester, MA USA

†Current affiliation: Shire HGT; Lexington, MA USA

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Abbreviations: sORF, single open reading frame; HC, heavy chain; LC, light chain; Int, intein; SP, signal peptide; SP1, initial signal peptide; SP2, embedded signal peptide; PhoPol, *P. horikoshii* DNA Polymerase I intein; PfuLon, *P. furiosus* Lon intein; PabLon, *P. abysii* Lon intein; A18, A18 VκII signal peptide; HL, heavy chain-intein-light chain sORF configuration; LH, light chain-intein-heavy chain sORF configuration; CHO, Chinese hamster ovary; MTX, methotrexate

Efficient production of large quantities of therapeutic antibodies is becoming a major goal of the pharmaceutical industry. We developed a proprietary expression system using a polyprotein precursor-based approach to antibody expression in mammalian cells. In this approach, the coding regions for heavy and light chains are included within a single open reading frame (sORF) separated by an in-frame intein gene. A single mRNA and subsequent polypeptide are produced upon transient and stable transfection into HEK293 and CHO cells, respectively. Heavy and light chains are separated by the autocatalytic action of the intein and antibody processing proceeds to produce active, secreted antibody. Here, we report advances in sORF technology toward establishment of a viable manufacturing platform for therapeutic antibodies in CHO cells. Increasing expression levels and improving antibody processing by intein and signal peptide selection are discussed.

Introduction

The emerging success of monoclonal antibodies (mAbs) as therapeutic agents for a wide range of human diseases in the past decade has heightened both medical and commercial interests in further exploiting these molecules in the clinic. As a consequence, there has been increased motivation to find ways to both improve and maximize production of mAbs in the manufacturing process.[1,2]

Current technology used for the production of antibodies in mammalian cells involves transfection of DNAs encoding the heavy chains (HC) and light chains (LC) of antibodies, with each driven by separate regulatory elements. Protein expression is improved by employing methods that increase transcription efficiency and gene dosage.[3,4] Duplication of genetic elements within a single plasmid, however, may limit expression by introducing competition for transcription factors and enhancer-binding proteins. This may ultimately lead to problems that contribute to the genetic instability of these engineered cell lines.[5-8] Competition for transcription factors could be especially significant in cells with many copies of the expression vector. A variety of different molecular approaches have been taken to address these issues, although they resulted in varying degrees of success.[9-12]

In 2009, we described a novel technology using an intein-based expression vector for the production of mAbs.[13] Inteins are intervening protein sequences only found embedded in other proteins, with the flanking protein sequences referred to as exteins. Together with their associated exteins, inteins are transcribed as single mRNAs and translated as single polypeptides. Through autocatalysis, the intein is excised from the polypeptide with concomitant ligation of the exteins in the process known as protein splicing.[14,15] Modifications to native intein reactions have been previously investigated and used in biotechnology applications, but primarily for protein purification in vitro.[16-18]

In our initial studies, an expression cassette consisting of IgG1 HC-*Pyrococcus horikoshii PolI* intein-IgG1 LC was used to produce a single mRNA translated into a single polypeptide. This polypeptide was led into the endoplasmic reticulum via the first signal peptide (SP). As in normal protein splicing, the intein was excised from the polypeptide, but unlike normal splicing, the adjacent exteins (HC and LC) were not ligated together by

*Correspondence to: Wendy R. Gion; Email: wendy.gion@abbvie.com
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design. Liberated HC and LC were assembled and secreted as functional antibody. Although antibody was reliably produced and detected, our initial studies indicated only low levels of secreted antibody. Of particular interest was the processing of the second "embedded" SP that was presented out of context to the signal recognition particle.

In this report, we describe a broad-based application of our existing system for the enhanced production of mAbs. By conducting a systematic survey of intein chemistries and cleavage properties of the second, embedded SP, we demonstrate that use of certain enhancements such as intein and LC SP choice, construct architecture and expression of multiple antibody candidates resulted in production levels that were suitable for manufacturing in Chinese hamster ovary cells (CHO). This is the first report of antibody expression from a single open reading frame (sORF) vector that attains expression levels compatible with commercial manufacturing demands.

Results

Choice of intein, construct design and embedded signal peptide can be used to improve antibody expression. Efforts to increase antibody production from a single open reading frame focused on the choice of intein and embedded signal peptide. Inteins are classified primarily by function and vary widely in their amino acid sequence. Different classes of inteins were therefore likely to perform differently in the context of the antibody expression construct. In addition, changes made to the adjacent extein residues directly affect the intein's capability to cleave or splice.[16] With this in mind, we created a panel of constructs to determine the optimal intein options and which placement of the heavy and light chain "exteins" would be the most efficient to achieve high expression of correctly assembled and functional antibody. Furthermore, we observed retention of the antibody light chain signal peptide in our previous study, which indicated that the signal peptide in secreted antibody could be varied to support expression. Substitution of another embedded signal peptide was used to improve signal sequence processing.

The general structure of our sORF construct is as follows: An initial signal peptide (SP1), N-terminal extein (e.g., IgG1 HC, intein, a second "embedded" signal peptide (SP2) and finally the C-terminal extein (e.g., IgG1 LC). Although the intein is a native amino acid sequence, the non-native N and C-terminal exteins drive the reaction toward cleavage, but, by design, away from splicing. We tested a limited intein library with diverse polypeptide sequences and characteristics (**Table 1A**). We constructed vectors that contained, in a single open reading frame, varied placement of IgG1 HC and LC linked to intein and with or without altered SP2 (**Fig. 1**; the abbreviation HL denotes the antibody heavy chain -intein-antibody light chain, LH the light chain – intein-heavy chain structure, signal peptides placed as shown).

As we predicted, the choice of intein influences the processing of the antibody polypeptide. *PabLon* intein, coupled to antibody sequences in the HL orientation, processes HC and

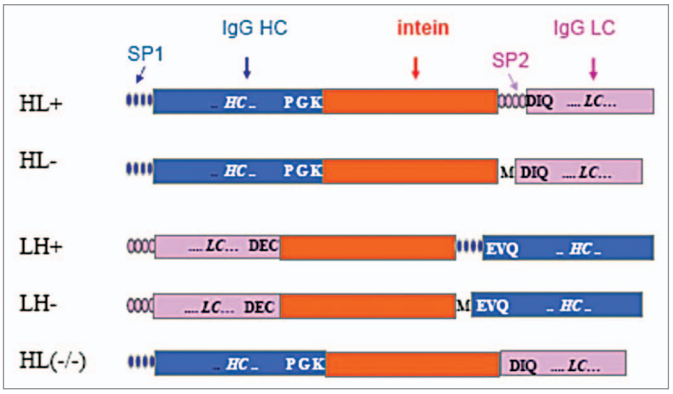

Figure 1. Schematics of sORF constructs. To maximize correct processing of IgG heavy and light chains, several sORF constructs were designed. The schematics portraying intein, signal peptides and alternative orientations of heavy and light chain sequences are shown. Heavy and light chain coding regions display the three amino- or C-terminal residues adjacent to the intein. The sequence between the intein and C-terminal Ig domain has been noted as a signal peptide (S.P. ovals), a methionine residue (M) or no intervening sequence.

LC to expected sizes. The LH orientation resulted in reduced processing of the polypeptide complex. Other inteins gave partial processing to mature antibody chains (*MjaKlbA, PchPPRB*) or failed to cleave from the adjacent HC (*PabKlbA*) as seen in **Figure 2B**. Western blots indicate intracellular expression of six major protein species with variations between constructs (**Fig. 2A and B**). The upper band present on the blot corresponded to unprocessed polyprotein precursor (HC-int-LC, ~90–114 kDa depending on the intein) and was recognized by anti-HC and anti-LC antibodies. In the PabLon HL–, PabLon LH– and PFuLon HL– lanes, a smaller, unidentified band is visible directly under the largest band. This may represent a degradation product. A band corresponding to intein linked to HC was present at the expected size of ~67 to 70 kDa and was only recognized by anti-HC antibody (*MjaKlbA* and *PabKlbA*). A band corresponding to intein linked to LC was present at the expected size of ~42 kDa and was only recognized by anti-LC antibody (*MjaKlbA* and *PchPRP8*). A band corresponding to LC with its SP was present at the expected size of 26 kDa in HL+ samples (HL(+) versions of *MjaKlbA* and *PabKlbA*). Finally, a HC band (50 kDa) and a LC band (24 kDa) were present in cells transfected with either sORF or positive control 2-vector system where HC and LC were expressed on separate plasmids. The use of conventional vectors produced only HC and LC bands (**Fig. 2C and D**). Removal of SP2 resulted in incomplete C-terminal cleavage of intein from LC (**Fig. 2D**), resulting in inefficient processing and secretion of antibody. The *PhoPol* and *PfuLon* inteins coupled with the HL orientation of antibody chains resulted in near-complete processing as judged by western blot.

The presence of separated HC and LC demonstrated cleavage from intein at both N-terminal and C-terminal junctions. These data suggested the choice of intein and SP2 sequence directly affected N-terminal and C-terminal processing from HC and LC, as well as the intracellular expression level.

Table 1A. Comprehensive expression ranking and figure location for each construct

SP1	Extein 1	Intein	SP2	Extein 2	Expression (Secreted)	Orientation	Outcome	Figure
3–23	HC	PabLon	A18	LC	+++	HL+	High expression and high % SP2 correctly cleaved from LC	2, 3, 4
3–23	HC	PabLon	A26	LC	+++	HL+	Incomplete processing	2
3–23	HC	PabLon	A17	LC	+++	HL+	Incomplete processing	2
3–23	HC	PabLon	L2	LC	++	HL+		2
3–23	HC	PabLon	B2	LC	++	HL+		2
3–23	HC	PabLon	A27	LC	++	HL+		2
3–23	HC	PabLon	A23	LC	++	HL+		2
3–23	HC	PabLon	A19	LC	++	HL+		2
3–23	HC	PabLon	A14	LC	++	HL+		2
3–23	HC	PabLon	L20	LC	+	HL+		2
3–23	HC	PabLon	L25	LC	+	HL+		2
3–23	HC	PabLon	B3	LC	+	HL+		2
3–23	HC	PabLon	Met	LC	+++	HL–	Met retained on LC	1
3–23	HC	PfuLon	Met	LC	+++	HL–	Met retained on LC	1
3–23	HC	MjaKlbA	Met	LC	+++	HL–	Intein retained on HC	1
3–23	HC	PabKlbA	Met	LC	+++	HL–	Intein retained on HC	1
L5	LC	PabLon	Met	HC	++	LH–		1
3–23	HC	PabLon	L5	LC	++	HL+		1
3–23	HC	PfuLon	L5	LC	++	HL+		1
L5	LC	PfuLon	Met	HC	++	LH–		1
L5	LC	PhoPol	Met	HC	++	LH–		1
L5	LC	PabLon	3–23	HC	+	LH^+		1
L5	LC	PfuLon	3–23	HC	+	LH^+		1
3–23	HC	PhoPol	Met	LC	+	HL–		1
L5	LC	PhoPol	3–23	HC	+	LH^+		1
3–23	HC	PhoPol	L5	LC	+	HL+		1
3–23	HC	PhoPol	-	LC	Not detected	HL–/–		1
3–23	HC	All other inteins tested	L5	LC	+/++	HL+		1
3–23	HC	All other inteins tested	Met	LC	+/++	HL–		1
L5	LC	All other inteins tested	3–23	HC	+/++	LH^+		1
L5	LC	All other inteins tested	Met	HC	+/++	LH–		1

The single ORF construct diagram is displayed above the table and each component is labeled. Exteins 1 and 2 are either IgG heavy chain or IgG light chain. Intein refers specifically to those included in the intein library and can be found in Part B of this table. GenBank accession numbers are given. Intein sequences can be found in **Table S3.** The Signal peptide 2 is SP2 and its sequences can be found in Part C of this table. Table columns correspond to the following categories: Columns (1–5) Components; Column (6) Expression level from a scale of + to +++ (with + referring to lowest expression and +++ referring to highest expression); Column (7) Orientation of extein sequences (as referred to here, exteins are either heavy chain or light chain, thus orientation is either HL or LH) with or without signal peptide 2 (+ or –); Column (8) Outcome (given only for best expressers) and Column (9) Associated figure. In order to choose inteins with a wide variety of characteristics the New England Biolabs intein database, InBase (www.neb.com/neb/inteins.html), was surveyed and a range of inteins chosen according to species, size and distinguishing features. The chosen inteins were used in the constructs denoted in **Figure 1.** In order to improve signal peptide 2 processing from mature light chain, VBase (http://vbase.mrc-cpe.cam.ac.uk/) was surveyed and a range of native human kappa light chain signal peptides chosen.

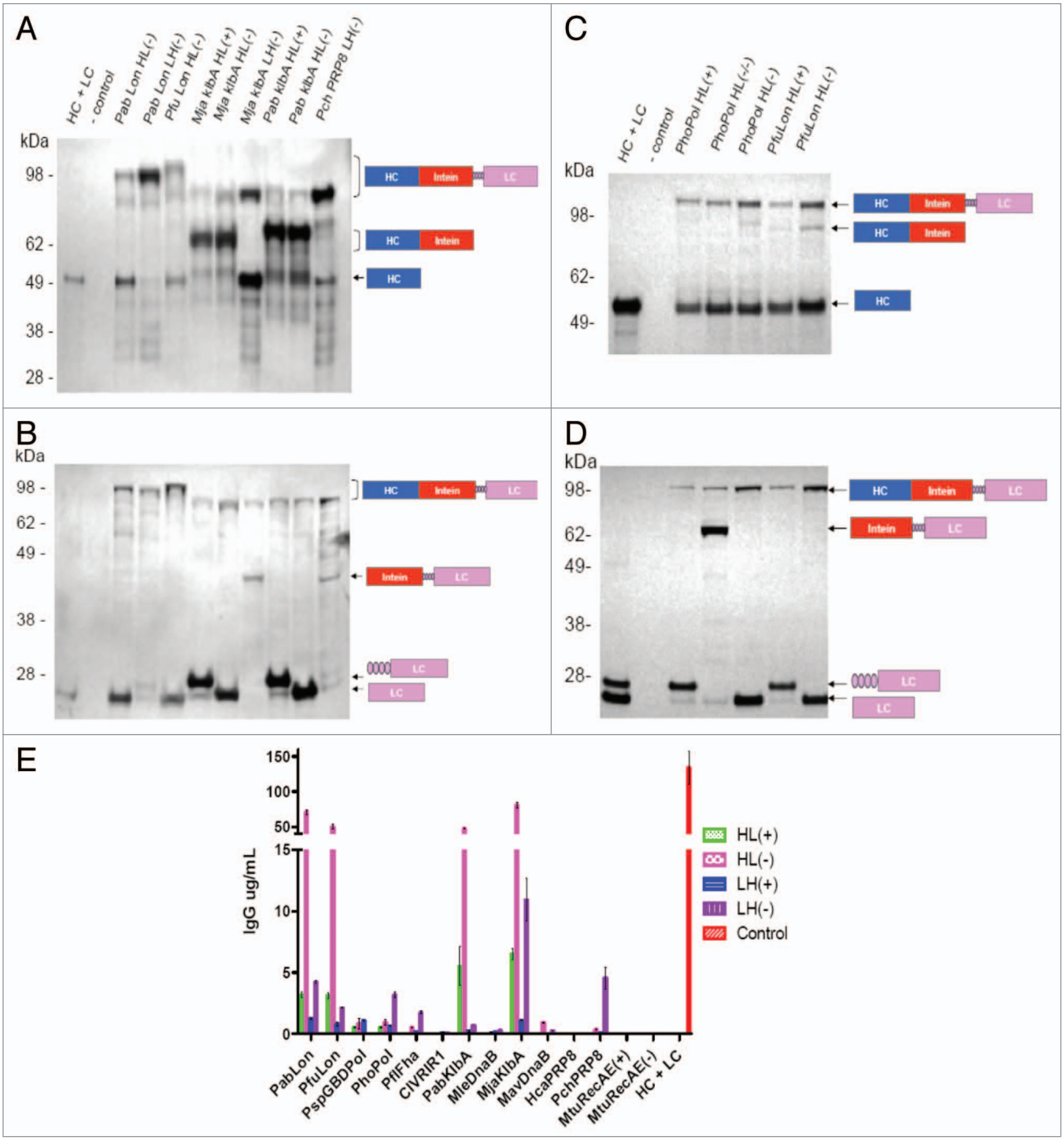

Figure 2. Effect of Intein choice, construct design and light chain signal peptide alterations on IgG1 expression levels and intracellular processing. HEK293 cells were transfected with the above ORFs containing the library of inteins (**Table 1B**) in the expression vector pTT3. Cell pellet samples were separated on SDS-PAGE and analyzed by western blots for IgG1 heavy chain (**A, C**) and for kappa light chain (**B, D**). Figures to the right of the blots describe the molecular nature of the bands based on mobility. HC, antibody heavy chain protein; LC, antibody light chain protein. Antibody expression from separate ORFs was analyzed in Lane 1 of all the western blots (HC+LC). Culture supernatants were collected on day 8 post transfection, and their IgG levels were measured by ELISA (**E**).

IgG ELISA results indicate the original sORF construct using *PhoPol* intein produced modest amounts of secreted antibody that would be useful for small scale studies,[13] while an increase in secreted antibody was seen with the substitution of *PfuLon, PabLon, PabklbA, MjaklbA* or *PchPRP8* inteins for *PhoPol*, particularly in the HL– orientation (**Fig. 2E**).

Table 1B. Limited intein library and distinguishing characteristics

Intein	Species	Size(aa)	Phylogenetic Domain	Accession #
PhoPol	**Pyrococcus horikoshii**	**460**	Archaea	BA000001.2:1686361
PabLon	Pyrococcus abyssi	333	Archaea	NC000868.1:1543460
PfuLon	Pyrococcus furiosus	401	Archaea	AE009950.1:481472
PabKlbA	Pyrococcus abyssi	196	Archaea	NC_000868.1:1346260
MjaKlbA	Methanococcus jannaschii	168	Archaea	NC_000909.1: 701559
PspGBDPol	Pyrococcus species GBD	537	Archaea	AAA67132.1
PchPRP8	Penicillium chrysogenum	161	Eucarya	AM042015
HcaPRP8	Histoplasma capsulatum	534	Eucarya	HCAG_04562
PflFha	Pseudomonas fluorescens	148	Eubacteria	AAY90835
CIVRIR1	Chilo Iridescent Virus	339	Eucarya	T03053
MtuRecAE+ (Mtu-H37Rv RecA)	Mycobacterium tuberculosis	440	Eubacteria	P0A5U4.1
MtuRecAE- (Mtu-H37Rv RecA minus endonuclease region)	Mycobacterium tuberculosis	137	Eubacteria	P0A5U4.1
MleDnaB	Mycobacterium leprae	145	Eubacteria	CAA17948.1
MavDnaB	Mycobacterium avium	337	Eubacteria	AAK09265

Specifically, a ~70 fold increase in secreted antibody was seen in PabLonHL– over PhoPolHL-, demonstrating a crucial advance toward a larger scale manufacturing enabled process.

Therefore, we demonstrated that certain intein construct architectures could produce extensive increase in antibody expression, thus demonstrating the effect of the combination of intein choice, extein placement and SP2 alteration on secreted antibody levels.

Modification of SP2 to improve LC processing. Having improved secreted antibody expression through intein choice, extein placement and SP2 alteration, we next focused on producing correctly processed HC and LC in the context of the PabLonHL construct. In HL– as in **Figure 1A**, methionine was retained on the LC N-terminus. In HL+, HC processes correctly from SP1, while LC processing from SP2 is incomplete. We refined LC processing through substitution of SP2 in PabLonHL+ with other human V kappa LC SPs.

A limited collection of human LC SPs was assembled (**Table 1B**). ELISA results (**Fig. 3A**) revealed that four of the 13 Vk SPs had antibody titers greater than 10 μg/mL. These were further purified using Protein A, analyzed by cation exchange chromatography and compared against traditionally produced IgG1 (**Fig. 3B**). The construct containing VkII A18 SP produced a profile similar to the IgG1 standard. Mass spectrometry (**Fig. 3C**) revealed that most of the purified antibody from the A18 construct was correctly processed. A summary of antibody processing and expression levels produced by various constructs is shown in **Table 1C**.

Pablon HL(A18) configuration applied to different antibody isotypes. To further demonstrate the utility of the sORF platform, PabLon HL(A18) constructs were prepared for three antibodies, a different IgG1 kappa LC antibody; an IgG2; and IgG1 lambda LC antibody. These are referred to as Antibodies 2, 3 and 4 in **Figure 4**, respectively. The original PabLonHL(A18) IgG1 was used as a control (Antibody 1 in **Fig. 4**). In addition to the isotype, all four antibodies differed in target specificity, reflecting the different complementarity-determining regions in the antibodies.

Table 1C. The chosen signal peptides were substituted for SP2 in the construct PablonHL(+) and are also shown in **Figure 1**

Signal Peptide	Isotype	Amino Acid Sequence
3–23	VH3	MEFGLSWLFLVAILKGVQC
Control (L5)	Vκl	MDMRVPAQLLGLLLLWFPGSRC
A14	VκVI	MVSPLQFLRLLLLWVPASRG
A17	VκII	MRLPAQLLGLLMLWVPGSSG
A18	VκII	MRLPAQLLGLLMLWIPGSSA
A19	VκII	MRLPAQLLGLLMLWVSGSSG
A23	VκII	MRLLAQLLGLLMLWVPGSSG
A26	VκVI	MLPSQLIGFLLLWVPASRG
A27	VκIII	METPAQLLFLLLLWLPDTTG
B2	VκV	MGSQVHLLSFLLLWISDTRA
B3	VκIV	MVLQTQVFISLLLWISGAYG
L2	VκIII	MEAPAQLLFLLLLWLPDTTG
L20	VκIII	MEAPAQLLFLLLLWLTDTTG
L25	VκIII	MEPNKPQHSFFFLLLLWLPDTTG

Poros A results (**Fig. 4A**) revealed varied secreted antibody levels, but no correlation was found between expression level and isotype. Northern blot analysis confirmed each antibody was transcribed as a single mRNA species (~3750 bp) that hybridized with both HC and LC probes (**Fig. 4B**). No individual HC or LC transcript was detected, in contrast to samples that expressed HC and LC using two vectors. In these samples, mRNAs for the HC (~1.4 kb) and the LC (~0.7 kb) were detected instead. Western blots confirmed LC SP cleavage efficiency was similar between constructs and produced the same species as described earlier (**Fig. 4C and D**).

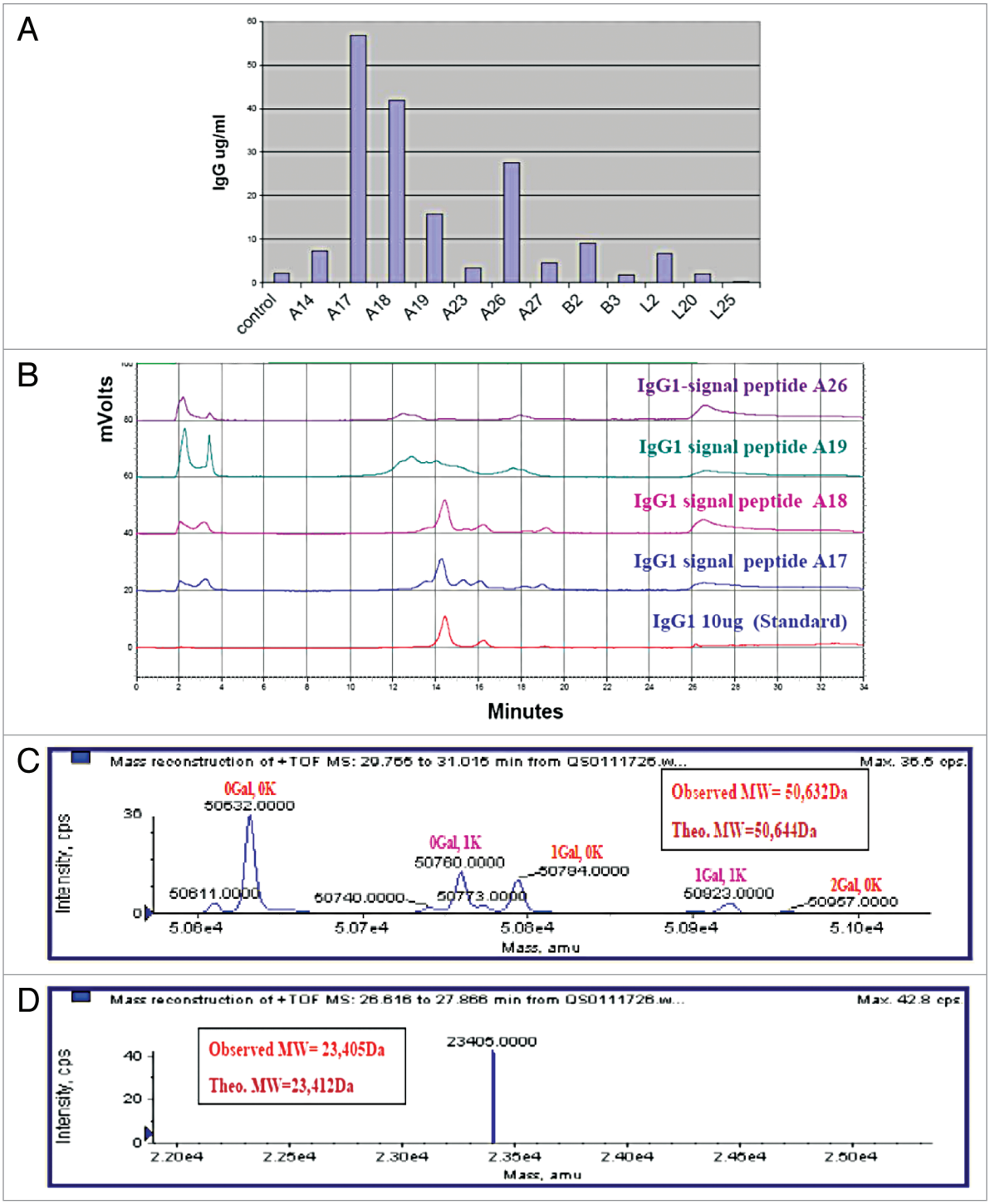

Figure 3. Effect of light chain signal peptide on IgG1 expression levels and intracellular processing. (**A**) To maximize correct processing of IgG heavy and light chains in the context of the PabLon intein in the HL orientation, Vkappa signal peptides (**Table 1C**) were inserted before the light chain coding region in the HL(+) constructs and transfected into HEK293 cells. Culture supernatants were collected on day 8 post transfection, and their IgG levels were measured by ELISA. (**B**) Protein A affinity purified IgGs were analyzed by cation exchange chromatography. PabLonHL(A18) exhibited a similar chromatogram as the IgG1 control and was further analyzed by mass spectrometry for heavy chain (**C**) and light chain (**D**).

Antibody sizes and binding properties are comparable. Antibodies secreted using PablonHL(A18) constructs described above were analyzed by mass spectrometry under reducing conditions (**Fig.** 5). The analysis identified molecular species that correlated with the predicted molecular weight of each LC with a minor peak corresponding to LC with A18 SP associated. Although western blots detected an intracellular intein-LC species, this was absent from the final secreted product. The

molecular weight of HC was as expected for all samples (data not shown).

Antibodies produced using PablonHL(A18) constructs were analyzed for binding to antigen using surface plasmon resonance (SPR). The kinetic on-rate, off-rate and overall affinities were determined using 1–100 nM antigen concentrations (**Table 2**). These data indicated that antibodies produced from PablonHL(A18) sORF constructs have antigen affinity that is similar to the same antibodies that are produced by conventional vectors, confirming they are functional and correctly folded.

Antibody production in a CHO stable cell line using PablonHL(A18) architecture. To confirm that the sORF system functions in commercially-important CHO cells, antibody derived from a scaled down manufacturing process was tested for processing and binding function. A stable CHO cell line was generated using the sORF PablonHL(A18) vector, and comparisons were made against an existing stable CHO cell line that was created using a conventional vector. The level of screening done to isolate the sORF PablonHL(A18) CHO line was comparable to that used to develop the conventional production cell line. Cells were grown under batch-fed conditions and samples taken on days 8–11. The IgG titers were determined by Poros A. The stable sORF CHO cell line produced ~1 g/Liter at the time of harvest vs. ~1.2 g/Liter from the conventional CHO cell line (**Fig. 6A**). Day 11 supernatants were harvested and Protein A purified. Purified antibody was analyzed by mass spectrometry and determined to be 80–90% correctly processed (**Fig. 6B**). Biacore analysis demonstrated similar kinetic rate parameters and overall affinity between the sORF and the conventionally-produced purified antibody (**Table 3**).

Discussion

Robust expression of therapeutic antibodies from mammalian cells is a priority for the pharmaceutical industry. Predominantly, expression cassettes are designed that stably integrate into the host cell genome. Much research has been done to improve vector design, including ways to amplify copy number, facilitate genomic integration and incorporate epigenetic insulation elements.[3,19,20,21] Methods using co-expression of chaperones and protein disulfide isomerase (PDI) to aid in multi-subunit protein assembly and secretion have resulted in varying outcomes.[22,23]

Current vector systems are impeded by differential expression from heavy and light chain coding regions when these are delivered on separate plasmids. When both coding regions are present on a single vector, a 1:1 ratio of heavy to light chain still cannot be guaranteed due to the existence of mechanisms such as promoter occlusion[24] and translational interference.[25] Even when a single promoter is used in conjunction with an IRES sequence, differential transcription of the coding regions, with the second gene being transcribed at a lower level, is observed.[26,27] In contrast, by using a sORF vector, one heavy and one light chain are connected by an intein, transcribed as a single mRNA, translated as a single polypeptide and then subsequently cleaved through autocatalytic activity of the intein, resulting in a 1:1 ratio of heavy to light chain. In addition, the use of an expression cassette for sORF-produced antibody is simpler than using vectors with two sets of regulatory sequences and could potentially result in better chromosomal integration and genetic stability.

We have previously described the sORF vector system containing the *PhoPol* intein for generation of secreted, active antibody in the context of IgG1 kappa expression.[13] This novel approach produced antibody of similar quality to that made with conventional vectors, but, although the yield was suitable for small scale expression, it was insufficient for use in large scale manufacturing. This was not due to translation of sORF mRNA because intracellular antibody protein levels appeared to be comparable to the traditional expression method. Cleavage of intein from heavy and light chain without splicing also appeared efficient, with a minimal amount of unprocessed polypeptide remaining. The inefficient cleavage of light chain signal peptide, however, led to limited secretion of correctly folded antibody. We postulated that this was due to the unique presentation of the second signal sequence by the sORF polypeptide, rendering it only partially identifiable by signal peptidase.[28] Briefly, it is believed that sORF polypeptide inserts into the endoplasmic reticulum via the first signal peptide, possibly resulting in uptake of the entire molecule before autocatalytic cleavage of the intein. In this model, the second signal peptide would be presented to the signal peptidase embedded in the polypeptide as opposed to a free N-terminus. Although it would become unmasked after cleavage from the intein, the signal peptide may not be fully recognized at this point, possibly due to its location in the lumen of the ER or conformation of the peptide.

In this study, we used a more systematic approach to assess antibody production utilizing the sORF vector design. Initially, we tested a limited, but diverse, panel of inteins in combination with different placements of IgG1 heavy and light chain to render the area flanking the second signal peptide more amenable to signal peptidase cleavage. Since inteins have no strong homology except for key residues, a survey was done of InBase for inteins, whether verified or theoretical, that contained differences in certain amino acids. Both the *PfuLon* and *PabLon* intein lacked the highly conserved penultimate histidine and were postulated to have an alternative splicing mechanism, much like klbA inteins.[29] In addition, since in the original construct, *PhoPol* efficiently cleaved from the methionine present at the N-terminus of the light chain signal peptide, we concluded that methionine was favorable to the cleavage reaction. This feature was incorporated into the resulting design of HL– and LH– orientations. It was thought that methionine aminopeptidase present in eukaryotic cells would process the liberated N-terminus, leaving either mature light or heavy chain.[30] While this was not the case, most likely due to the presence of either glutamic acid or aspartic acid in the +1 position of mature heavy and light chain, respectively, expression levels increased dramatically in the HL– orientation with both *PfuLon* and *PabLon* inteins (**Fig. 2E**).

Although HL– produced high titer functional antibodies, the residual methionine on mature light chain was viewed as a disadvantage because it may potentially lead to a higher

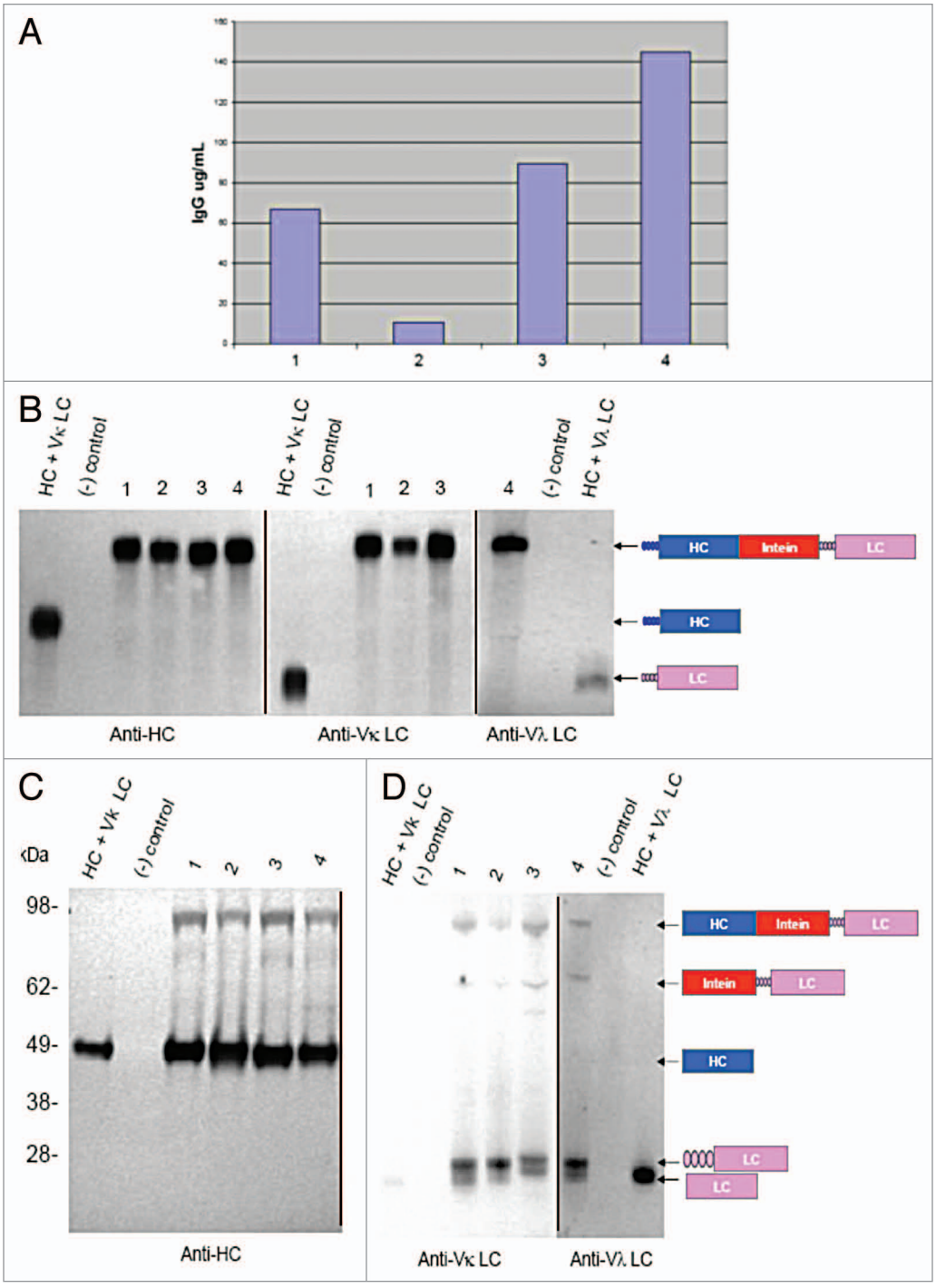

Figure 4. IgG secretion of different antibody isotypes using the PablonHL(A18) sORF vector design. To verify that sORF design was applicable across a range of antibody types, PabLonHL(A18) expression vectors containing different antibody types (1 = IgG1 kappa light chain, example 1; 2 = IgG1 kappa light chain, example 2; 3 = IgG2 and 4 = IgG1 lambda light chain) were constructed and transfected into HEK293 cells. (**A**) Culture supernatants were collected on day 8 post transfection and IgG levels measured by Poros A. (**B**) Northern blots of total RNA from transfected cells were hybridized with DNA probes against heavy chain or light chain (Vκ and Vλ). Note that the panels shown are from three separate hybridizations with the respective DNA probes indicated below. (**C, D**) Cell pellet samples were analyzed by western blot with antibodies for IgG1 heavy chain and for kappa and lambda light chains. Figures to the right of the blots describe the molecular nature of the bands based on mobility. HC, antibody heavy chain protein; LC, antibody light chain protein; int, intein. Note that the panels shown are from three separate western blots with the respective detecting antibodies indicated below.

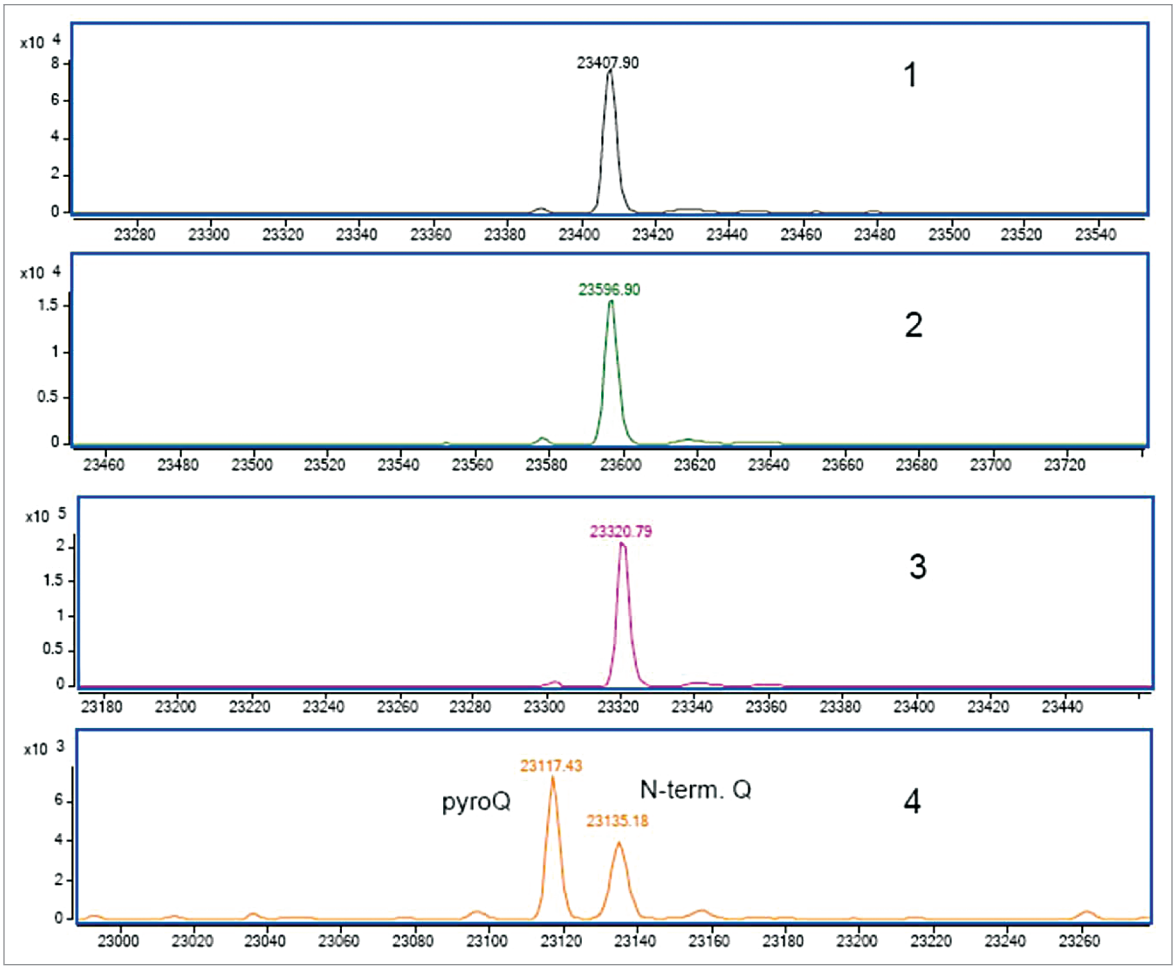

Figure 5. Molecular weight analysis of light chain from different antibody isotypes using the PablonHL(A18) sORF vector design. Day 8 post transfection supernatants were purified by Protein A affinity and analyzed by reduced mass spectrometry, light chain only shown. One through 4 correspond to samples described in **Figure 4**.

immunogenicity of the therapeutic antibody. To improve processing of light chain and produce antibody identical to that generated by conventional techniques, we tested a limited selection of other native human V kappa light chain signal peptides (http://vbase.mrc-cpe.cam.ac.uk/) in place of the original light chain signal peptide in the PablonHL+ construct. Of the signal peptides that allowed high level expression, VκII A18 produced a processing profile most similar to conventional antibody, with the majority of antibody found to be fully processed post-purification.

The demonstration of increased expression levels with four different antibodies confirmed that this phenomenon was not isotype dependent and was broadly applicable across mAbs. With this substitution of VκII A18 for the existing light chain signal peptide, we increased the expression level of our control IgG1 from 2.0 ug/mL to ~65.0 ug/mL in the HEK293 transient transfection system.

Ultimately, to prove that the sORF system can be used as a basis for a viable manufacturing platform, we created a stable CHO cell line expressing IgG1 using a DHFR-containing sORF expression vector with methotrexate amplification. Under bioreactor-like, fed-batch conditions, cultures produced ~1 g/Liter of secreted antibody, an expression level well within the realm of manufacturability. IgG1 was 80–90% correctly processed post-Protein A purification. Additional purification steps will remove the unprocessed product based on the increased hydrophobicity of the retained signal peptide.

In these studies, we demonstrated functional antibody expression at levels comparable to established technologies using intein-based sORF vectors that represent a novel approach in this field of research. The data presented here clearly demonstrate this vector design's feasibility as an antibody manufacturing platform.

Materials and Methods

Vector construction. The cloning of and vector construction with *P. horikoshii* DNA Polymerase I intein (PhoPol) has been previously described.[13] The vector pTT3-HCintLC-0aa used is referred to as PhoPolHL+, and the vector pTT3-HCintLC-0aa-K used is referred to as PhoPolHL(–/–) in this publication (see

Table 2. Antibody -antigen binding kinetics of different antibody isotypes expressed using the PablonHL(A18) sORF vector design, as determined by surface plasmon resonance

Antibody	1		2		3		4	
Vector	**sORF**	**Conv.**	**sORF**	**Conv.**	**sORF**	**Conv.**	**sORF**	**Conv.**
Kinetic on-rate, k_a ($M^{-1}s^{-1}$)	1.6×10^6	1.7×10^6	2.2×10^5	2.3×10^5	8.0×10^5	8.9×10^5	1.7×10^6	1.8×10^6
Kinetic off-rate, k_d (s^{-1})	1.6×10^{-4}	1.1×10^{-4}	6.9×10^{-5}	7.0×10^{-5}	9.6×10^{-3}	9.7×10^{-3}	1.0×10^{-5}	1.4×10^{-5}
Overall affinity K_D(M)	9.9×10^{-11}	6.4×10^{-11}	3.1×10^{-10}	3.1×10^{-10}	1.2×10^{-8}	1.1×10^{-8}	1.1×10^{-11}	7.7×10^{-12}

Conv. = conventional separate ORF expression heavy and light chains.

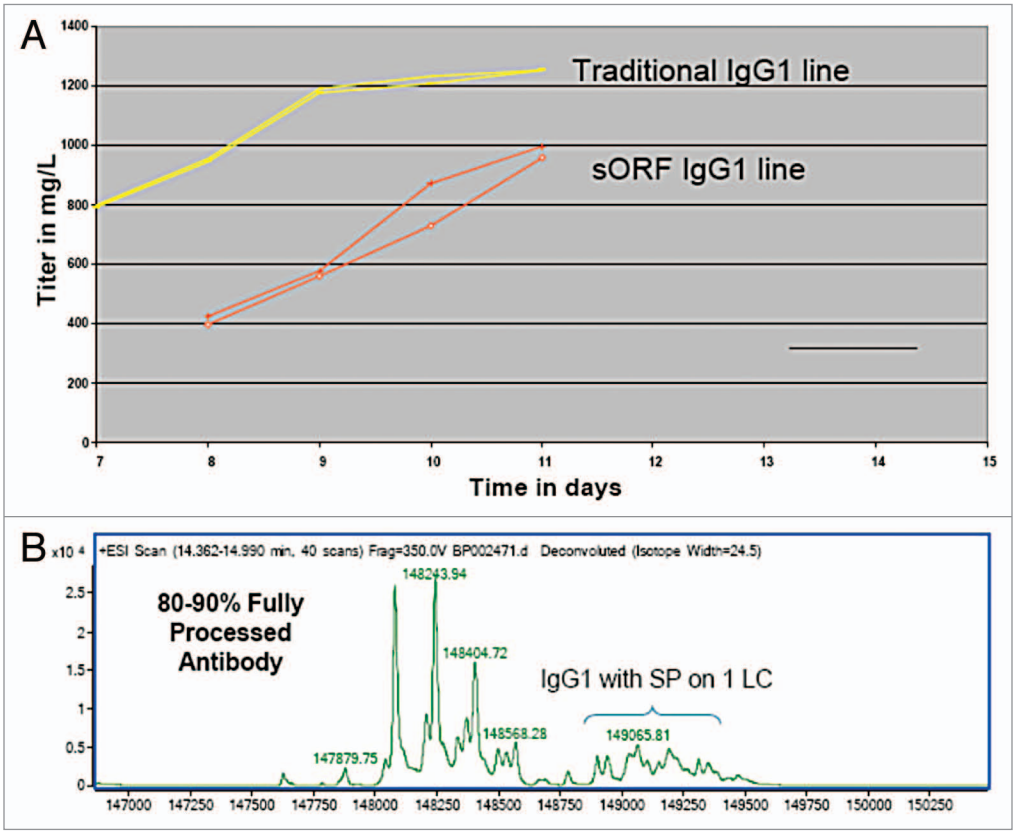

Figure 6. Expression of antibodies in CHO using the PablonHL(A18) architecture under bioprocess conditions. (**A**) In order to demonstrate sORF can be produced under bioprocess conditions at a level suitable to manufacturing, CHO cells producing IgG1 from a PabLonHL(A18) expression vector were grown under fed batch conditions in shake flasks and IgG levels measured on days 8–11 by Poros A. (**B**) Supernatants harvested on day 11 were purified by Protein A affinity and analyzed by mass spectrometry.

Fig. 1A for construct diagrams). Primer sequences and pairing for each construct can be found in **Tables S1 and S2**. Inteins are designated using the first 3 letters of the organism followed by the protein of origin. Thus, the *P. abysii* Lon intein is referred to as PabLon.

P. furiosus genomic DNA (ATCC, cat.# 43587D-2) was used as a template to amplify the *PfuLon* intein (GenBank accession #AE009950.1:481472) using primers Pfu-lon-5' and Pfu-lon-3'. The pTT3 vector backbone fragment containing IgG1 HC and LC was generated by PCR, using primers pTT3int-HR5' and pTT3int-HR3'. The *PfuLon* intein was fused to the above IgG1 antibody HC at its 5' end and the antibody LC to its 3' end by overlapping PCR reactions, with two different junctions, using primers Pfu-lon-HR5' and Pfu-lon-HR3' to yield the vector pTT3-Pfu-lon-HL(+). PCR was performed using Platinum Taq High Fidelity Supermix (Life Technologies, cat.# 12532–016).

To create the construct pTT3-PfuLonHL– a DNA fragment was generated using pTT3PfuLonHL+ as a template and PCR amplification was performed using primers lon-minus-SP-3' and lon-minus-SP-5'. The fragment was self-ligated using the Ligate IT Rapid Ligation Kit (Affymetrix, cat.# 78400) to produce pTT3-PfuLonHL–.

Intein survey library. To create the intein survey orientation library, the inteins listed in **Table 1A** were selected from Inbase, a publicly curated database sponsored by New England Biolabs (www.neb.com/neb/inteins.html). DNAs codon-optimized for mammalian expression were synthesized by GeneArt (**Table S3**). Each intein was tested in four configurations. The intein was either fused to the IgG1 antibody HC at the intein's 5' end and the antibody LC to the intein's 3' (HL) end or to the antibody LC at the intein's 5' end and the antibody LC to the intein's 3' end (LH). The designation of (+) or (–) refers to the presence of a SP or a methionine, respectively, 3' to the intein (see **Fig. 1** for construct diagrams). Constructs PfuLon HL+ and HL– are described above.

Kappa aignal aequences library. Creation of the LC SP library in the expression vector, pTT3-PabLonHL– was accomplished in two stages: creation of the new SP junction region in an intermediate vector and then the transfer of the junction regions to the expression vector. A PCR fragment containing the intein and vector sequences was prepared from plasmid PabLon-Xcm-BsiW_pENTR/D-TOPO using primers 10852 and 9482. This fragment is common to all constructs. Individual LC fragments containing new kappa SPs were amplified using an Ultramer (IDTdna, Coralville, Iowa) that encodes a short overlap with the intein fragment 3'-end, the new SP and 5'-end of mature coding sequence and the primer 9842 in flanking vector. All PCR products were treated with DpnI (New England Biolabs, cat.# R0176S)and purified by Qiaquick PCR Purification kit (Qiagen, cat.#28104). Vector and insert fragments were mixed, transformed into competent One Shot Top10 *E. coli* (Life Technologies, cat.# C4040–10) screened by colony PCR and sequenced. Correct inserts were identified and reamplified from the colonies with primers 10868/10869 that provide overlap at the XcmI and BstZ17I sites. The fragments were recombined into pTT3-PabLonHL– that was prepared by BstZ17I (New England Biolabs, cat.# R0594S) and XcmI (New England Biolabs, cat.# R0533S) digestion as described above. Transformants were screened by colony PCR and sequenced for correct clones. DNA maxiprep stocks were prepared using the Hi-Speed Plasmid Maxi Prep Kit (Qiagen, cat.# 12662) and the entire expression region including promoter and polyA tract were confirmed by sequence analysis.

A18-antibody libraries. To create the PablonHL(A18) constructs containing an IgG1 lambda LC antibody, the *PabLon* intein was fused to the above IgG1 antibody HC at its 5' end and the antibody LC to its 3' end by overlapping PCR reactions, with two different junctions, using primers pTT3–351HC-F, 351HC-Pab-R, Pab-A18–351LC-F and 351-pTT3-R. The primer Pab-A18–351LC-F introduced the A18 sequence at the 5' end of the mature LC. The vector, pTT3, was prepared by digestion with restriction endonucleases PmeI (New England Biolabs, cat.# R0560S) and NotI (New England Biolabs, cat.# R0189S) and treatment with calf intestinal alkaline phosphatase (New England Biolabs, cat.# M0290S). This removed the vector polylinker and left the flanking vector promoter region and polyA regions identical to the previous intein constructs. The insertion of the three PCR products was performed simultaneously by mixing them with the prepared vector and transformation of competent One Shot Top10 *E. coli* cells (Life Technologies, cat.# C4040–10). Transformants were screened by colony PCR and sequenced for correct clones.

Table 3. Antibody -antigen binding kinetics of IgG1 expressed using the PablonHL(A18) sORF vector design in CHO under bioprocess conditions

Vector	sORF	Conventional
Kinetic on-rate, k_a ($M^{-1}s^{-1}$)	2.1×10^6	2.1×10^6
Kinetic off-rate, k_d (s^{-1})	1.6×10^{-4}	1.7×10^{-4}
Overall affinity, K_D (M)	7.3×10^{-11}	8.3×10^{-11}

To create the PabLonHL(A18) constructs containing an IgG2 kappa LC antibody, the *PabLon* intein was fused to the above IgG2 antibody HC at its 5' end and the antibody LC to its 3' end by overlapping PCR reactions, with two different junctions, using primers TT3–5xxHC-F, 5xxHC-Pab-R, Pab-A18–595LC-F and 595LC-TT3-R. The primer Pab-A18–595LC-F introduced the A18 sequence at the 5' end of the mature LC. pTT3 vector preparation, combination with PCR products, transformation and the colony screen used was the same as above.

To create the PabLonHL(A18) constructs containing an IgG1 kappa LC antibody different from that previously described, the *PabLon* intein was fused to the above IgG1 antibody HC at its 5' end and the antibody LC to its 3' end by overlapping PCR reactions, with two different junctions, using primers TT3–5xxHC-F, 5xxHC-Pab-R, Pab-A18–510LC-F and 510LC-TT3-R. The primer Pab-A18–510LC-F introduced the A18 sequence at the 5' end of the mature LC. pTT3 vector preparation, combination with PCR products, transformation and the colony screen used was the same as above.

To create the PabLonHL(A18) CHO expression construct containing the original IgG1, the vector, pJV, was prepared by digestion with restriction the endonucleases NruI (New England Biolabs, cat.# R0192S) and NotI (New England Biolabs, cat.# R0189S). This removed the vector stuffer fragment and left the flanking vector promoter region and polyA regions. The pTT3-PabLonHL(A18) construct was also prepared by digestion with the restriction endonucleases NruI and NotI to excise the sORF HC-int-LC cassette. The vector and insert were combined using standard ligation techniques and transformation of One Shot Top10 competent *E. coli* cells (Life Technologies, cat.# C4040–10). Screening of the transformants was done by restriction digestion and candidates sequenced.

Expression in HEK293–6E cells. Expression vectors were introduced into HEK 293 cells as previously described.[31] Briefly, cells in exponential growth phase were transfected with 0.25–1.0 μg/mL plasmid DNA by adding a complex solution of plasmid DNA and PEI (polyethylenimine linear, 25 kDa) (Polysciences, cat.# 24765–2) at a ratio 1:2. After 20–24 h, Tryptone N1 medium (Tekni-Science, cat.# 19553) was added to a final concentration of 0.5%. Culture volumes were 25 mL. Cell pellet samples were harvested on day 3 to 5 for analysis by northern blot and western blot. Conditioned media was harvested on day 8 for analysis by ELISA and Protein A purification.

Expression in CHO cells. Expression vectors were introduced into a DHFR-deficient CHO host cell line B3.2, a subclone of

the DUXB11 line[32] by calcium phosphate transfection.[33] Stable transfectants were established under nucleoside-deficient growth conditions. Antibody expression in the resulting cell lines was enhanced by selection in increasing concentrations of methotrexate (MTX, Sigma-Aldrich, cat.# M8407), a process referred to as amplification.[34-36] Cell lines producing the highest amount of antibody under 100 nM MTX selection were subcloned by limiting dilution. The subclones with the highest antibody expression levels were then adapted to grow in suspension in serum-free medium. Suspension culture volumes were 200 mL.

Northern blot analysis. Total RNA was isolated from cell pellets, separated on formaldehyde-agarose gels blotted using labeled DNA probes using standard methods. Probe templates used are the coding regions of the antibody HC and LC, and they were labeled with Alkaline Phosphatase and detected using the AlkPhos Direct Labeling and Detection with CDP-Star Reagents kit (GE Healthcare, cat.# RPN3690).

Western blot analysis. For the analysis of intracellular polyprotein and antibody expression, cells were lysed by heating in 1X NuPAGE LDS Sample Buffer (Life Technologies, cat.# NP0007) with NuPAGE Sample Reducing Agent (Life Technologies, cat.# NP0004). Samples were separated on SDS-PAGE gel, transferred to nitrocellulose and blotted with antibodies against human IgG (Thermo Scientific, cat.# 31413), human kappa LC (Thermo Scientific, cat.# 31414) or human lambda LC (Thermo Scientific, cat.# 31131). Peroxidase conjugated AffiniPure Rabbit Anti-Goat IgG, Fc fragment was used as a secondary antibody as needed (Jackson Immunoresearch Laboratories, cat.# 305–035–046). ECL western blotting substrate (Thermo Scientific, cat.# 32209) was used for detection.

Analysis of antibody secreted into culture supernatant. ELISAs were performed using standard methods, using Goat Anti-Human IgG, UNLB (Southern Biotech cat.# 2048–01)and Goat Anti-Human IgG/HRP (Southern Biotech, cat.# 2048–05), 2% milk in PBS as a blotting buffer and K-Blue (Neogen, cat.# 03011) as a substrate. Plates were read with a Spectramax microplate reader at a primary wavelength of 650 nm and a reference wavelength of 490 nm. The secreted antibody was affinity purified with standard methods using Protein A agarose beads (Life Technologies, cat.# 15918–014), Immuno Pure (A) IgG Binding Buffer (Thermo Scientific, cat.# 54200), PBS at pH 7.4 as a wash buffer and 0.1 M acetic acid/150 mM NaCl at pH 3.5 as an elution buffer (neutralized using 1 M Tris pH 9.5). N-terminal amino acid sequences were obtained using the standard Edman method at Proseq.

Analysis of antibody integrity by mass spectrometry. Molecular weights of antibody samples were analyzed by LC-MS. A 1200 capillary HPLC system (Agilent) with a protein microtrap (Michrom Bioresources) was used to desalt and introduce samples into a 6510 QTOF mass spectrometer (Agilent). A gradient was run with buffer A (0.08% FA and 0.02% TFA in HPLC water) and buffer B (0.08% FA and 0.02% TFA in acetonitrile) at a flow rate of 50 μL/min to elute the samples. For determination of HC and LC molecular weight the samples were reduced using 20 mM DTT at 37°C for 30 min.

Analysis of antibody charge heterogeneity using cation exchange chromatography. The charge heterogeneity of antibody samples was assessed by Cation Exchange Chromatography. A HPLC system (Shimadzu) with binary pumps was employed with a Dionex ProPac WCX-10 column (Thermo Scientific, cat.# DX054993). Buffer A was 10 mM Na_2HPO_4 at pH 7.5 and Buffer B was 10 mM Na_2HPO_4, 500 mM NaCl at pH 5.5. The antibody charge variants were eluted by a linear gradient from 6% to 16% of Buffer B in 22 min, then from 16–100% of B in 5 min and remaining for 2 min and back to equilibrium at 6% of Buffer B until 34 min. The flow rate was 1 mL/min and the column oven temperature was at 30°C. Samples were diluted to 0.1 mg/mL to reduce the salt content and 10 ug–50 μg of the samples were injected onto the column. A UV detector with a wavelength of 280 nm was used to monitor proteins. A CHO-derived IgG standard was analyzed for comparability.

Surface plasmon resonance analysis of antibody binding to antigen. Real-time binding interactions between antibody captured across a CM5 biosensor chip via immobilized goat anti-human IgG Fc and a soluble ligand were measured using a Biacore 3000 or T200 instrument (GE LifeSciences, Pittsburgh, Pennsylvania). Briefly, antigen aliquots were diluted into a HBS-EP+ (GE LifeSciences, cat.# BR1006–69) buffer and were injected across the captured antibodies as well as a reference surface (immobilized goat anti-human IgG only) at a flow rate of 50 μl/min for 5 min to determine the on rate. Dissociation time was specific for each antigen antibody pair (**Fig. S1**). The sensor chip surface was regenerated between cycles with one 30 sec injection of 10 mM glycine at pH 1.5, at 100 μL/min. The resulting experimental binding sensorgrams were then evaluated using either the BIA evaluation 4.0.1 (Biacore 3000) or Biacore T200 Evaluation software version 1 to determine kinetic rate parameters. Data sets for each antagonist were fit to a 1:1 binding model (global fit analysis protocol while selecting fit locally for maximum analyte binding capacity, R_{max}, attribute). In this case, the software calculated a single dissociation constant (k_d), association constant (k_a) and affinity constant (K_D).

Disclosure of Potential Conflicts of Interest

WG, RD, DR, EF, LM, LS, SB, AI, BP, CC, JM and GC are AbbVie employees. YK is a former AbbVie employee. The design, study conduct and financial support for the research was provided by AbbVie. AbbVie was responsible for the interpretation of data, review and approval of the manuscript.

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Supplemental Material

Supplemental materials may be downloaded here:
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