Transient expression of human antibodies in mammalian cells

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Recombinant expression of antibody molecules in mammalian cells offers important advantages over traditionally utilized bacterial expression, including glycosylation required for antibody functionality and markedly reduced levels of endotoxin contamination. Advances in transient mammalian expression systems enable high yields (>100 mg/liter) that now allow for effective recombinant antibody production at a reasonable cost. Here, we provide step-by-step protocols for the design and recombinant expression of full-length IgG antibodies and antibody-derived constructs (including Fab, Fc-fusions and bispecifics) in mammalian cells. Antibody constructs are designed by combining antibody variable domains, generated by phage display or derived from human/humanized monoclonals, with constant regions. The constructs are then expressed from mammalian vectors, secreted into culture media, purified by affinity chromatography and characterized by biolayer interferometry. This article provides detailed protocols, sequences and strategies that allow the expression and purification of endotoxin-free antibody reagents suitable for testing in animal models within a 3-week time frame.

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INTRODUCTION

The recombinant production of antibodies and antibody-derived constructs can present challenges, particularly in the small-to-medium scale that is commonly required for academic research. This is largely due to problems with expressing such constructs in bacteria such as *Escherichia coli*, which historically has been the dominant system for the expression of recombinant proteins in academic settings. Such production challenges can result in low-quality antibody preparations, often with detrimental effects on the quality of subsequent biological experiments[1,2].

Although small antibody fragments (such as single-domain and single-chain variable fragments (scFvs)) can be readily expressed in bacteria, yields for larger antibody constructs (including fragment antigen binding (Fab), fragment crystallizable (Fc)-fusion proteins, bispecifics and immunoglobulin G (IgG)) are generally poor[3]. Moreover, expression in bacteria results in a lack of glycosylation (leading to reduced immune effector functions)[4–6] and can result in the contamination of purified antibodies with bacterial endotoxins[7]. Such contamination has the potential to activate innate immune cells[8], complicate the interpretation of cell-based assays[9] and result in pyrogenic effects following *in vivo* administration[10,11].

In an effort to address these limitations, a range of eukaryotic expression systems for the production of antibodies constructs have been developed, including expression systems in yeast and insect cells[12]. However, glycosylation patterns in these cells differ substantially from those observed in mammals[13], limiting their use in preclinical research and in the development of human biopharmaceuticals[14]. Such limitations do not apply to recombinant proteins produced in mammalian cells, such as human embryonic kidney 293 (HEK293) and Chinese hamster ovary (CHO) cells[15]. Although stable cell lines can be generated in both systems[16], the process tends to be time-consuming and has, for academic purposes, been largely superseded by the increased efficiency of transient expression, particularly when the production of several candidates is required.

Although initially limited to biopharmaceutical companies, suspension-adapted and serum-free mammalian expression systems are now becoming more broadly available to academic research groups. Recently developed methods rely on culture media capable of supporting high cell densities ($>1 \times 10^7$ cells/ml) and utilize transcriptional enhancers, leading to improved recombinant yields that range from tens to hundreds of milligrams per liter of culture[17,18]. Notably, a number of studies report >1 g/liter pre-purification yields for specific recombinant antibodies[19,20]. This substantial increase in yields enables effective production of glycosylated recombinant antibody reagents in an academic setting. The increase in yields also results in a considerable reduction in cost, from a strategic perspective narrowing the cost disadvantage of mammalian expression with bacterial culture (due to longer expression times, more expensive media, cost of transfection reagents and higher capital investment). The protocols outlined here are thereby likely to improve both direct (i.e., more efficient production) and indirect aspects (i.e., more efficient downstream usage) of antibody development pathways.

Protocol overview and potential applications

This protocol outlines detailed strategies for the design (**Box 1**) and transient expression (Steps 23–29) of full-length IgG, Fab fragment, Fc-fusion and bispecific antibody constructs (**Fig. 1**). Sequences for constant antibody domains are provided (**Supplementary Data 1**), with variable domain sequences derived from humanized/human monoclonals or generated using selection approaches (such as phage display)[21]. The protocol also includes detailed steps for the purification of recombinant antibody reagents secreted into culture medium using protein A/G/L affinity chromatography (Step 30A) or immobilized metal ion affinity chromatography (IMAC, Step 30B). Finally, methods for the detection and removal of endotoxin contaminant are outlined

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Box 1 | Design of antibody constructs for mammalian expression

Several considerations must be taken into account when designing gene constructs for mammalian expression. First, restriction sites for cloning into a suitable expression vector should be included in the design. These sites must be absent in the insert and present within the multiple cloning site of the selected expression vector. As an example, we typically introduce a KpnI restriction site at the 5′ end and a BamHI restriction site at the 3′ end of designed constructs (**Supplementary Data 2–8**) for cloning into the pCEP4 expression vector (Steps 1–16). Key requirements for efficient expression of transgenes include the introduction of a Kozak sequence[52], an N-terminal signal peptide (allowing for secretion into media), and a C-terminal stop codon. Human antibody variable domains (V_H and V_L) mediate binding to antigens and their sequences can be derived from antibodies of known specificity. Alternatively, novel specificities can be selected using a range of selection technologies, including phage display and the immunization of animals in combination with humanization strategies[34–36,53,54].

As outlined in **Figures 2** and **3**, IgG, Fab and Fc-fusion formats contain antibody constant domains (C_L, C_H1, C_H2, C_H3). The amino acid sequences for these domains can be obtained from publicly available databases (such as UniProt (http://www.uniprot.org); see **Supplementary Data 1**) and back-translated into DNA. It is recommended to utilize appropriate mammalian codon tables when performing this step, in order to optimize expression. Following back-translation, constructs should be tested for sequence complexity and necessary adjustments should be made in order to avoid repetitive DNA sequences (IDT's codon optimization online tool is particularly useful for performing the steps described above, http://www.idtdna.com/codonopt). After the design is finalized, constructs are generated by gene synthesis using commercial suppliers. Standard preparations of cloning vectors containing designed inserts (4 µg of plasmid DNA) are sufficient for performing this protocol.

An important consideration for the design of IgG and Fc-fusions is whether the Fc region (i.e., the C_H2 and C_H3 domains) is derived from human or mouse domains. This is of particular importance if the therapeutic activity of the expressed constructs will be assessed in mouse models of disease. The Fc regions of human and mouse IgG differ in their ability to engage murine Fc-gamma receptors (FcγR) and consequently display differential levels of Fc-mediated immune effector functions (antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)) in mice[55]. Importantly, immune effector functions mediated by Fc can influence the therapeutic efficacy of antibodies[56]. Therefore, if the contribution of Fc-mediated effector functions is to be fully appreciated in mouse models, it is recommended that Fc regions originating from murine IgG2a (for use in Balb/c mice) or IgG2c (for use in C57BL/6 mice) be used. Alternatively, Fc regions derived from the human IgG1 isotype can be used if experiments are to be performed in FcγR-humanized mice[57] or in immunodeficient mice engrafted with human immune cells[58]. If Fc-mediated immune effector functions are undesirable for a specific application, mutations can be introduced into mouse IgG2a/c and human IgG1 Fc regions in order to disrupt FcγR binding and/or complement activation (**Supplementary Data 1**).

The introduction of a C-terminal hexahistidine (6×HIS) tag allows for the purification of expressed constructs via immobilized metal affinity chromatography (IMAC; Step 30B). This is particularly useful in instances in which protein A/G/L affinity chromatography is not feasible, and it adds versatility to the purification process. This is particularly the case for Fab fragments, which lack the antibody Fc region and cannot be purified by protein G chromatography. Because of their monovalent nature, Fab fragments are particularly suited for the determination of binding affinities and for structure determination by X-ray crystallography[3]. By including a hexahistidine tag in the heavy chain, but not the light chain, of Fab constructs (**Fig. 3**), transfection of excess light chain, followed by IMAC purification, will ensure that only fully assembled Fab is purified.

Information required for designing the above-described constructs, including example amino acid and DNA sequences, can be found in **Supplementary Data 1–8**.

(Steps 36–44), and we provide instructions for determination of antibody affinity using biolayer interferometry (BLI; Steps 45–57). Potential applications for antibody reagents produced following this protocol include structural, affinity determination and preclinical animal studies.

Advantages of the approach

This protocol provides steps for the expression of recombinant antibodies in mammalian cells. It relies on commercially available reagents and requires minimal optimization. More specifically, advantages over other comparable methods are as follows:

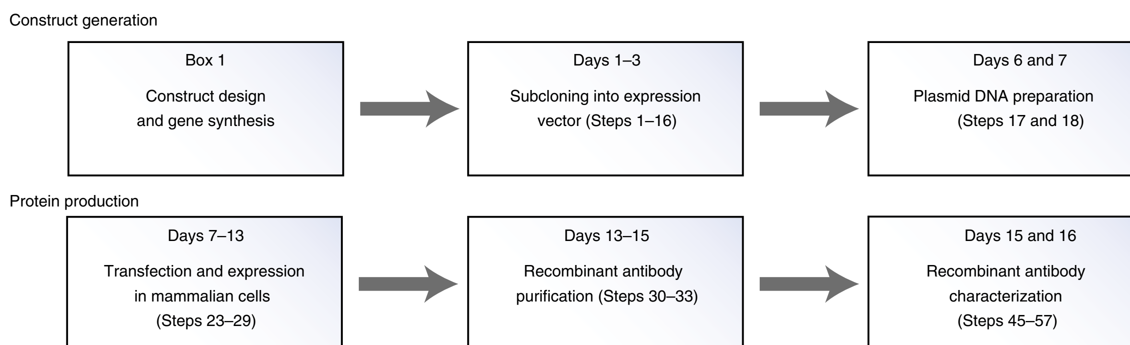

Figure 1 | Schematic overview of the procedure. Schematic representation of the steps required for the design and production of endotoxin-free recombinant antibody reagents by means of transient expression in mammalian cells.

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First, in our protocol, the production of the designed DNA constructs is carried out by gene synthesis and is outsourced to specialized companies. This takes advantage of technological advances driving a rapid decline in the cost of DNA synthesis[22,23], and removes much complexity and a source of potential errors in the protocol. For other applications, for instance when producing a large number of antibody variants of the same format, direct cloning into vectors containing desired antibody constant domains may still be more effective[24–26]. Second, we describe the expression of antibody heavy and light chains from two separate vectors, rather than from a single bicistronic vector relying on internal ribosomal entry sites (IRES) or self-processing peptides to simultaneously produce both antibody chains[27,28]. Although bicistronic constructs require less labor in terms of plasmid DNA preparation, they are not amenable to heavy-to-light chain transfection ratio optimization[29] and can become impractical when screening several pairings of different heavy chain and light chain mutants.

Limitations of the approach

This protocol makes use of cationic lipids as transfection reagents. These compounds display the highest levels of transfection efficiency but are more expensive than alternative transfection reagents[30]. The cost of transfection reagents can become apparent in the context of large-scale transfections. In our experience, sufficient amounts of recombinant antibody for structural and mouse preclinical studies are typically obtained from small- to medium-scale transfections (<500 ml of cell culture). If a larger scale of transient expression is required, researchers should consider the implementation and optimization of comparable methods, making use of the inexpensive cationic polymer transfection reagent polyethyleneimine[31]. Once a lead antibody candidate is selected for further development, the investment of time and resources for the generation of a stable mammalian cell line expressing the antibody of interest should be considered[32]. Alternatively, expression from stable transfection pools can be utilized if large quantities of the desired antibody are required in a relatively short period of time[33].

Experimental design

Expression constructs. In the first part of the PROCEDURE, gene constructs encoding IgG, Fab, Fc-fusion proteins or bispecific scFv are designed *in silico* (**Box 1**; **Figs. 2** and **3** and **Supplementary Data 1**) and obtained from companies offering gene synthesis services. Sequences of human variable regions are derived from high-affinity clones selected using phage display, as described in Lee *et al.*[21], or from humanized/human monoclonals[34–36]. Antibody constant region sequences are obtained from online databases (**Supplementary Data 1**). For expression in mammalian cell lines, antibody constructs are first recloned into suitable expression vectors. In this protocol, we make use of the pCEP4 expression vector, which relies on the widely utilized human cytomegalovirus (CMV) intermediate–early promoter/enhancer to drive overexpression of cloned inserts[37].

Antibodies and antibody-derived molecules. Example sequences of DNA constructs required for the production of different antibody formats are provided (**Supplementary Data 1–8**). The bivalent full-length IgG antibody format (**Supplementary Data 2** and **3**) has a long *in vivo* half-life and is particularly useful for performing preclinical studies in mice. In this context, important considerations regarding the contribution of Fc-mediated immune effector functions to antibody activity are discussed (**Box 1** and **Supplementary Data 1**). The monovalent Fab format (**Supplementary Data 4** and **5**) lacks the Fc antibody region, which reduces complexity for crystallography and affinity determination applications. As the Fc region is absent in Fab, these molecules are not necessarily amenable to protein A/G purification. Instead, a hexahistidine tag is incorporated into the Fab heavy chain to allow for efficient purification using IMAC (Step 30B). Genetic fusion of small proteins or cytokines to the antibody Fc region allows for the expression of bivalent Fc-fusion proteins (**Supplementary Data 6**) with prolonged *in vivo* half-life and activity[38]. We also provide instructions for the production of monovalent Fc-fusion proteins. For this purpose, a construct coding for the desired Fc-fusion (**Supplementary Data 6**) is co-transfected with a construct encoding a His-tagged Fc (**Supplementary Data 7**). This is followed by purification by IMAC using sequential elutions with increasing imidazole concentrations (Step 30B(iv)). Finally, the bispecific (or tandem) scFv format (**Supplementary Data 8**) incorporates variable regions derived from two antibodies for recognition of two different antigens. Such bispecific antibodies can be useful for a variety of therapeutic applications[39], including neutralization of two different receptors on the same cell, and dual targeting of the CD3 T-cell receptor subunit and a tumor-associated antigen to facilitate tumor killing.

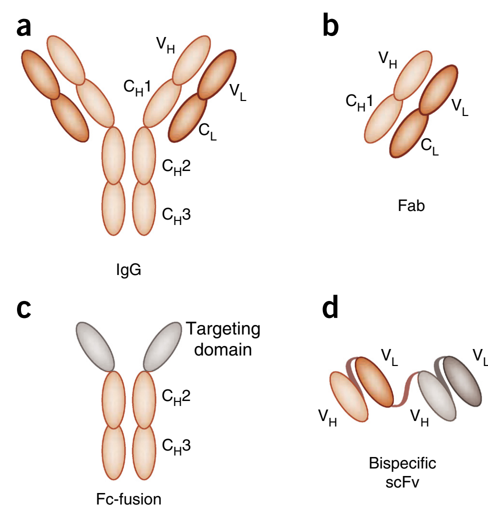

Figure 2 | Antibody formats for expression in mammalian cells (overview). (**a**–**d**) Schematic representation highlighting IgG (**a**), Fab (**b**), Fc-fusion (**c**) and bispecific scFv formats (**d**) with individual domains highlighted. C_H, constant heavy; C_L, constant light; V_H, variable heavy; V_L, variable light.

Antibody expression. Following large-scale preparation of plasmid DNA, we use a suspension-adapted HEK293 cell line (Expi293F) for high levels of transient expression. Optimization of heavy-to-light chain transfection ratios (IgG and Fab formats) is recommended for maximization of recombinant antibody yields. The inclusion of a positive-control Fab with known post-purification yield is also possible (100 mg/liter, **Supplementary Data 4** and **5**).

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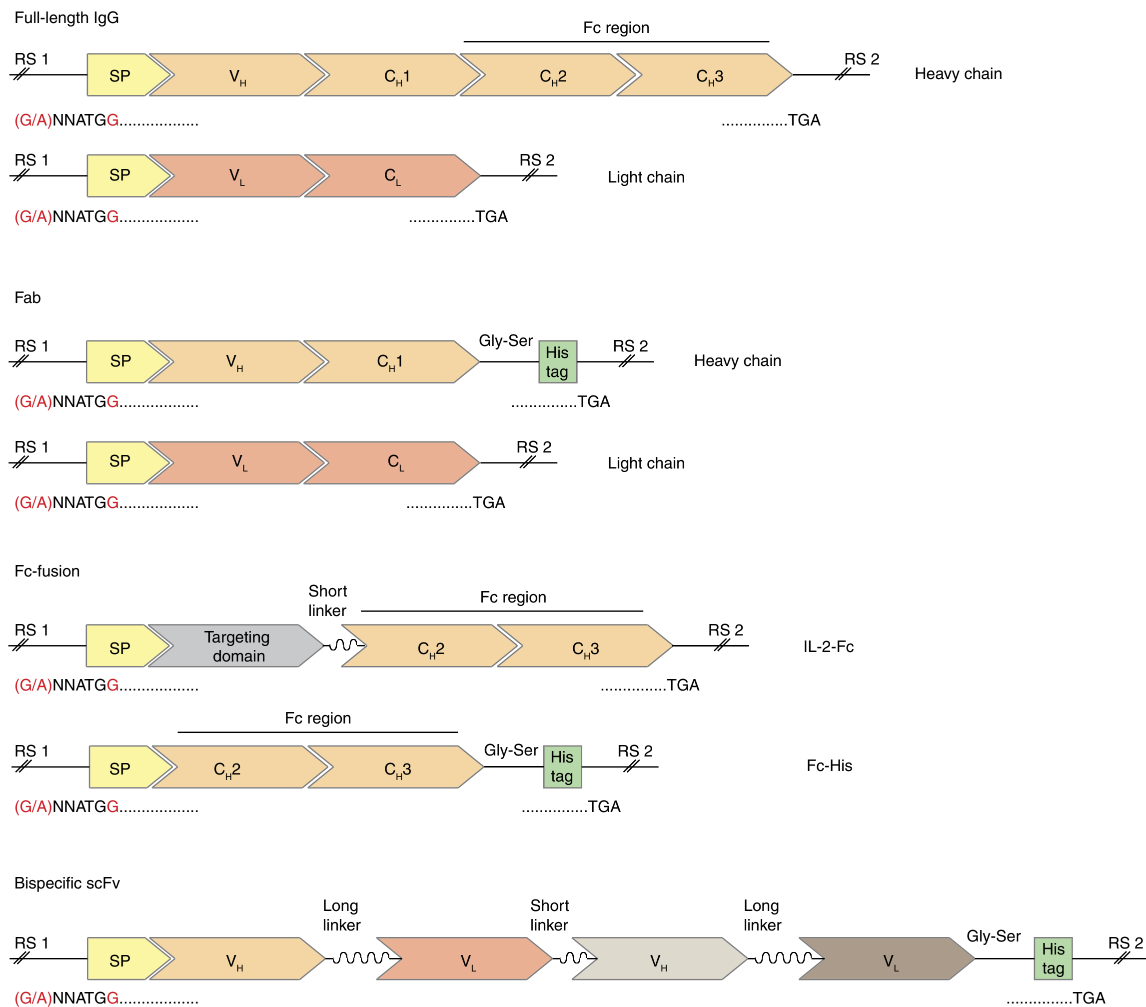

Figure 3 | Design of gene constructs. Schematic representation of the designed gene constructs coding for full-length IgG, Fab, Fc-fusion and bispecific scFv. Refer to **Supplementary Data 2–8** for fully annotated example construct DNA sequences. Kozak sequences at positions −3 and +4 are highlighted in red. RS, restriction site; SP, signal peptide; TGA, stop codon.

Box 2 | Endotoxin contamination in recombinant antibody preparations

Endotoxin content is an important consideration for purified antibody samples intended for use in *in vivo* models. Guidelines for studies can be based on the threshold pyrogenic human dose of 5 EU per kg per h[47]. For instance, a limit of 0.15 EU per h (i.e., 3.6 EU/d) can be assumed for a mouse weighing 30 g. Therefore, for example, a 100-µl daily injection (regardless of protein concentration) results an endotoxin content upper limit of 36 EU/ml (and, ideally, <10 EU/ml) for mouse studies. Although endotoxin concentration can be reduced by ion exchange chromatography (Steps 39–44), measures to minimize and prevent endotoxin contamination in the first place should be taken:

- Use plasmid DNA obtained from low-endotoxin preparation kits (such as the ZymoPURE Plasmid Maxiprep Kit) for transfection of mammalian cells.
- Monitor the mammalian expression culture for bacterial contamination and discard any contaminated preparations. Unusual culture appearance, low viability of Expi293F cells and presence of rod-shaped particles upon microscopic examination are indicative of bacterial contamination.
- Avoid the use of automated protein purification systems, especially if the system is also used to purify protein from bacterial cultures. If an automated system is used, sanitize with 1 M NaOH for at least 1 h before use.
- Use disposable, sterile, certified pyrogen-free containers for the entire mammalian culture, purification and storage process. This includes bottles used for storage of buffers and any materials used for dialysis of protein samples. Do not reuse storage containers, even if they have been autoclaved.
- Prepare elution and storage solutions using ultrapure water (Reagent Setup).
- Use aliquots of solutions to measure pH. Discard these aliquots after measurement.
- Filter all buffers using a 0.22-µm filter unit and store them at 4 °C for up to 6 months.

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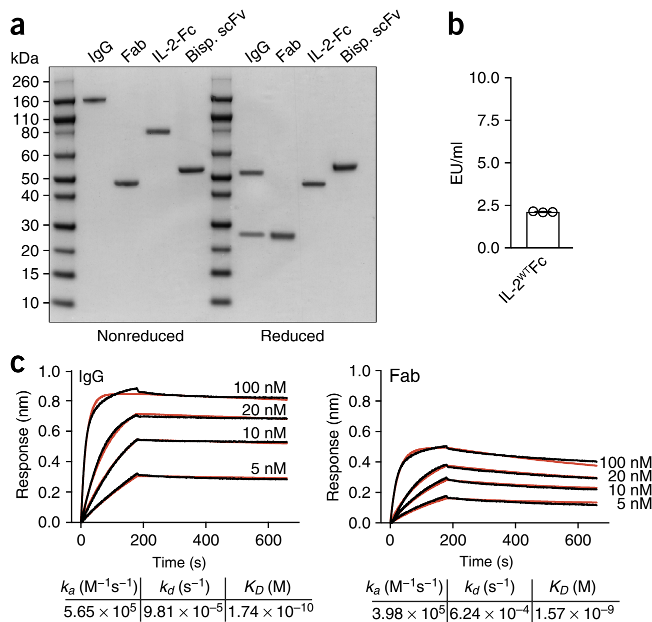

Figure 4 | Quality control of purified antibody preparations. (**a**) SDS-PAGE analysis of sample purity under nonreducing and reducing (10 mM DTT) conditions. Two micrograms of purified protein were analyzed using a NuPAGE 4–12% (wt/vol) Bis–Tris gel. Theoretical molecular weights are 146 kDa for Herceptin IgG (HC = 50 kDa, LC = 23 kDa), 47 kDa for Herceptin Fab (HC = 24 kDa, LC = 23 kDa), 84 kDa for IL-2-Fc and 53 kDa for bispecific scFv. (**b**) Quantification of endotoxin content in purified recombinant IL-2-Fc fusion protein[38]. The endotoxin content (in endotoxin units (EU)) of a ready-to-inject 168 μg/ml IL-2^{WT}Fc solution (2.1 EU/ml) was determined using the QCL-1000 colorimetric LAL assay (Lonza). The mean ± s.d. of technical triplicates is shown (coefficient of variation = 1.5%). (**c**) Biolayer interferometry analysis of produced Herceptin IgG and Herceptin Fab, displaying binding to immobilized HER2 antigen. Binding curves (black lines, 5–100 nM) were fitted using the 1:1 Langmuir model (red lines). Monovalent affinity for HER2 was determined from Fab fragment fits (K_D = 1.6 nM). Bisp., bispecific; HC, heavy chain; LC, light chain; WT, wild type.

Cell supernatants are harvested for purification of recombinant antibody reagent via protein A/G/L or IMAC. Although our approach typically yields highly pure recombinant antibody preparations after single-step purification, alternative protein purification strategies may be utilized if required[40].

Endotoxin removal. After antibody purification, endotoxin levels of the preparations are determined (Steps 36–38). Preparations that exceed the endotoxin thresholds (typically, 10 endotoxin units (EU)/ml, see **Box 2**) are further purified by ion-exchange affinity chromatography, resulting in high-purity antibody and fusion protein preparations (**Fig. 4**).

Antibody characterization. Purified antibodies can be characterized by a large variety of methods. In this protocol, we describe procedures for SDS-PAGE analysis (under both nonreducing and reducing conditions) (Step 31) and BLI (Steps 45–57). BLI is a widely used approach for testing antibody–antigen affinity and is based on the measurement of interference patterns between waves of light on a biosensor surface[41]. BLI can be performed on several platforms, such as the BLItz and Octet systems (Pall ForteBio). There is also a wide range of biosensor surface chemistries available for capture of a ligand. Alternatively, binding kinetics of purified antibodies can be characterized by surface plasmon resonance (SPR). SPR is available on several platforms, such as the Biacore systems (GE Healthcare Life Sciences).

Regardless of the platform used, it is important to ensure that experiments are designed to measure the 1:1 binding kinetics of an interaction, devoid of all avidity effects, which generally lead to an overestimation of binding affinity (**Fig. 4c**). For example, a soluble full-length IgG interacting with an immobilized target protein represents 2:1 binding kinetics due to the bivalent nature of IgG. Thus, the current protocol describes methods to assess binding affinity using the monovalent Fab antibody format.

MATERIALS

REAGENTS

- Custom synthesized genes coding for designed antibody formats (e.g., IDT, Genscript, GeneArt, Biomatik, Twist Biosciences, SGI-DNA), typically delivered in cloning vectors such as pUC18, pUC19 or pUC57. See **Table 1**, **Box 1**, and **Supplementary Data 1–8**.
- Custom synthesized oligonucleotides (IDT); see **Table 1** for details.
- Mammalian expression vector (e.g., pCEP4, Thermo Fisher, cat. no. V04450)
- pCEP4 Herceptin Fab heavy chain construct (optional, **Supplementary Data 4**; plasmid available from the authors upon request)
- pCEP4 Herceptin Fab light chain construct (optional, **Supplementary Data 5**; plasmid available from the authors upon request)
- Phusion high-fidelity DNA polymerase (NEB, cat. no. M0530S)
- GoTaq DNA polymerase (Promega, cat. no. M3005)
- QIAquick PCR Purification Kit or similar (Qiagen, cat. no. 28106)
- KpnI-HF restriction enzyme (NEB, cat. no. R3142L)
- BamHI-HF restriction enzyme (NEB, cat. no. R3136L)
- DpnI restriction enzyme (NEB, cat. no. R0176L)
- Deoxynucleotide (dNTP) solution mix (NEB, cat. no. N0447L)
- Formamide, for molecular biology (Sigma-Aldrich, cat. no. F9037) **! CAUTION** Wear nitrile gloves, goggles and a lab coat when handling formamide.
- T4 DNA ligase (NEB, cat. no. M0202L)
- Calf intestinal alkaline phosphatase (NEB, cat. no. M0290L)
- Ampicillin (Sigma-Aldrich, cat. no. A9518)
- Glycerol (Sigma-Aldrich, cat. no. G5516)
- 10× DPBS solution (Gibco, cat. no. 14200-166; see Reagent Setup)
- Ultrapure water (sterile water for irrigation or similar; Baxter, cat. no. AHF7114)
- *E. coli* DH5-alpha competent cells (NEB, cat. no. C2987H)
- ZymoPURE Plasmid Midiprep or Maxiprep Kit or similar (Zymogen, cat. no. D4201 or D4202)
- Expi293F cells (Thermo Fisher, cat. no. A14527) **! CAUTION** Cell lines should be routinely authenticated and tested for mycoplasma contamination.
- Expi293 serum-free medium (Thermo Fisher, cat. no. A1435101) **▲ CRITICAL** Protect medium from light sources.
- ExpiFectamine 293 Transfection Kit (Thermo Fisher, cat. no. A14525)
- Opti-MEM I reduced serum medium (Thermo Fisher, cat. no. 31985062)
- DMSO, sterile (Sigma-Aldrich, cat. no. D2650) **! CAUTION** DMSO is highly toxic; wear gloves at all times and avoid contact with skin.
- rProtein A agarose beads (Acro Biosystems, cat. no. MA-0422-03)
- rProtein G agarose beads (Acro Biosystems, cat no. MG-0422-03)
- rProtein L agarose beads (Acro Biosystems, cat. no. ML-0422-01)
- Glycine (Sigma-Aldrich, cat. no. 241261)
- Tris base (Sigma-Aldrich, cat. no. 154563)

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- Sodium hydroxide solution, 10 M (Sigma-Aldrich, cat. no. 656054) **! CAUTION** Sodium hydroxide solution is caustic. Work in a fume hood, wear nitrile gloves, goggles and a lab coat.
- Hydrochloric acid solution, 37% (wt/wt) (Sigma-Aldrich, cat. no. 320331) **! CAUTION** Work in a fume hood, wear nitrile gloves, goggles and a lab coat.
- pH-indicator strips (Merck Millipore, cat. no. 1.09535.0001)
- TALON metal affinity resin (Clontech, cat. no. 635502)
- Imidazole (Sigma-Aldrich, cat. no. I2399) **! CAUTION** Imidazole is toxic. Wear nitrile gloves, goggles, a lab coat and a face mask when handling imidazole. Avoid contact with skin and inhalation.
- MES monohydrate (Sigma-Aldrich, cat. no. M5287)
- Cobalt chloride hexahydrate (Sigma-Aldrich, cat. no. 202185)
- NuPAGE 4–12% (wt/vol) Bis–Tris SDS-PAGE precast gel (Invitrogen, cat. no. NP0336)
- NuPAGE LDS sample buffer (4×) (Invitrogen, cat. no. NP0007)
- Novex Sharp prestained protein standard (Invitrogen, cat. no. LC5800)
- NuPAGE MES-SDS running buffer (20×) (Invitrogen, cat. no. NP0002)
- InstantBlue protein stain (Expedeon, cat. no. ISB1L)
- Endotoxin detection assay (QCL-1000 chromogenic LAL endpoint assay; Lonza, cat. no. 50-647U)
- EZ-Link NHS-PEG$_4$-Biotin (Thermo Fisher, cat. no. 21329)
- LiteSafe 0.5-ml black microcentrifuge tubes (Argos Technologies, cat. no. T7456-001)
- Streptavidin biosensors for BLI (Pall ForteBio, cat. no. 18-5019)
- Agarose for gel electrophoresis (Lonza, cat. no. 50004)
- 10× TBE buffer (Thermo Fisher, cat. no. B52)
- SYBR Safe DNA gel stain (Invitrogen, cat. no. S33102)
- Parafilm M (Sigma-Aldrich, cat. no. BR701605)
- Agar (BD Difco, cat. no. 281210)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- EDTA (Sigma-Aldrich, cat. no. EDS)
- Tryptone (BD Bacto, cat. no. 211699)
- Yeast extract (BD Bacto, cat. no. 212720)
- DTT (Sigma-Aldrich, cat. no. D9779)
- Liquid nitrogen
- Trypan blue (Sigma-Aldrich, cat. no. T8154)

EQUIPMENT

- Thermal cycler (Bio-Rad, model no. C1000 Touch or similar)
- Shaking incubator, 37 °C, 250 r.p.m., for *E. coli* DH5-alpha cells
- Tissue culture incubator, 37 °C, 5–8% CO_2, with humidified atmosphere, for Expi293F cells
- Orbital shaker, 125 r.p.m., for Expi293F cells (Infors Celltron model or similar)
- Plate shaker capable of ≥ 1,000 r.p.m. (optional, for expression in tissue culture plates)
- Refrigerated laboratory centrifuge (Heraeus Multifuge model ×3 or similar)
- Milli-Q water purification system (Millipore) (optional)
- UV–visible spectrophotometer
- Electrophoresis power supply (Bio-Rad PowerPac or similar)
- DNA gel electrophoresis chamber (Bio-Rad Mini-Sub or similar)
- XCell SureLock electrophoresis system (Invitrogen, cat. no. EI0001)
- HPLC system (GE Healthcare AKTA Purifier or similar) (optional)
- Surface plasmon resonance system (GE Healthcare, model no. Biacore T200 or similar) (optional)
- BLI system (ForteBio, BLItz model or similar) (optional)
- 125-ml, 250-ml and 500-ml disposable Erlenmeyer flasks with vented cap (Corning, cat. nos. 431143, 431144 and 431145).
- Cell-freezing container (Mr. Frosty or similar; Thermo Fisher, cat. no. 5100-0001)
- Round-bottom 14-ml sterile tubes (Corning, cat. no. 352059)
- Screw-cap cryotubes (Nunc, cat. no. 377267)
- 0.22-µm vacuum filter units (Corning, cat. no. 431097)
- SnakeSkin dialysis tubing (Pierce, cat. no. 68100)
- Pierce disposable plastic columns, 10 ml (Thermo Fisher cat. no. 29924)
- Zeba spin desalting columns, 7K MWCO, 2 ml (Thermo Fisher, cat. no. 89889)
- Amicon Ultra-15 centrifugal filter units, 10 kDa (Millipore, cat. no. UFC901024)
- HiTrap Capto Q 1-ml columns (GE Healthcare)
- Sterile syringes (BD)
- 0.22-µm syringe-driven filters (Millipore, cat. no. SLGV013SL; Sartorius, cat. no. 16534-K)
- Hemocytometer (Sigma-Aldrich, cat. no. Z359629)
- SnapGene or SnapGene Viewer software (GSL Biotech, http://www.snapgene.com)
- IDT Codon Optimization Tool (Integrated DNA Technologies, http://sg.idtdna.com/CodonOpt)

REAGENT SETUP

Ampicillin stock solution (100 mg/ml) Dissolve 1 g of ampicillin in 10 ml of deionized water. Filter the solution through a 0.22-µm filter. Prepare 1-ml aliquots of stock solution. Store them at −20 °C for up to 1 year. Thawed aliquots should be freshly diluted 1,000-fold in LB agar or LB medium (final ampicillin concentration = 100 µg/ml).

LB ampicillin agar plates Dissolve 15 g of agar, 5 g of NaCl, 10 g of tryptone and 5 g of yeast extract in 900 ml of deionized water. Adjust the pH to 7.5 and bring the final volume to 1 liter with deionized water. Autoclave the solution. Cool the solution to 50 °C and add 1 ml of 100 mg/ml ampicillin stock solution. Pour the solution into plates. The plates can be stored at 4 °C for up to 4 weeks. The plates should be dried in flow bench before use.

LB ampicillin medium Dissolve 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl in 900 ml of deionized water. Adjust the pH to 7.5 and bring the final volume to 1 liter with deionized water. Autoclave the solution. Cool the solution to room temperature (20–25 °C) and add 1 ml of 100 mg/ml ampicillin stock solution. The medium can be stored at 4 °C for up to 4 weeks.

Low-endotoxin PBS buffer Mix 100 ml of 10× DPBS (Thermo Fisher) with 900 ml of ultrapure water to make the PBS buffer. The buffer can be stored at 4 °C for several months.

Hydrochloric acid (5 M) Add 41.4 ml of 37% (wt/wt) hydrochloric acid (HCl) to 58.6 ml of ultrapure water. This can be stored at room temperature for several months. **! CAUTION** Work in a fume hood, slowly add acid to water, not water to acid; mix the solution gently; wear nitrile gloves, goggles and a lab coat.

Sodium hydroxide (1 M) Add 10 ml of 10 M sodium hydroxide (NaOH) solution to 90 ml of ultrapure water. This can be stored at room temperature for several months. **! CAUTION** Work in fume hood; add NaOH to water, not water to NaOH; mix the solution gently; and wear nitrile gloves, goggles and a lab coat.

TALON wash buffer Add 21.2 g of NaCl to 900 ml of low-endotoxin PBS buffer and dissolve. Adjust the pH to 8.0 using 1 M NaOH and bring the final volume to 1 liter with additional PBS (final NaCl concentration = 500 mM). Filter the solution through a 0.22-µm filter. The solution can be stored at 4 °C for several months.

TALON elution buffer Add 5.1 g of imidazole to 450 ml of TALON wash buffer and dissolve. Adjust the pH to 8.0 using 5 M HCl. Bring the final volume to 500 ml with additional TALON wash buffer (final imidazole concentration = 150 mM). Filter the solution through a 0.22-µm filter. The solution can be stored at 4 °C for several months. **! CAUTION** Wear nitrile gloves, goggles and a lab coat when handling 5 M HCl. **! CAUTION** Wear nitrile gloves, googles, a lab coat and a face mask when handling imidazole. Avoid contact with skin and inhalation.

TALON regeneration buffer Dissolve 2.1 g of MES monohydrate and 8.8 g of NaCl in 450 ml of ultrapure water. Adjust the pH to 5.0 using 1 M NaOH and bring the volume to 500 ml with ultrapure water. Filter the solution through a 0.22-µm filter. The solution can be stored at 4 °C for several months.

Glycine–HCl elution buffer (0.1 M glycine, 0.1 M NaCl, pH 2.7) Dissolve 7.5 g of glycine and 5.8 g NaCl in 900 ml of ultrapure water. Adjust the pH to 2.7 using 5 M HCl and bring the volume to 1 liter using ultrapure water. Filter the solution through a 0.22-µm filter. The solution can be stored at 4 °C for several months. **! CAUTION** Wear nitrile gloves, goggles and a lab coat when handling 5 M HCl.

Tris-HCl solution (1 M) Dissolve 24.2 g of Tris base in 180 ml of ultrapure water. Adjust the pH to 7.6 using 5 M HCl and bring the volume to 200 ml using ultrapure water. Filter the solution through a 0.22-µm filter. The solution can be stored at 4 °C for several months. **! CAUTION** Wear nitrile gloves, goggles and a lab coat when handling 5 M HCl.

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PROCEDURE

Subcloning and large-scale preparation of DNA constructts ● TIMING 7 d

1| Using ultrapure water, resuspend the designed gene-synthesized constructs (see Experimental design, **Box 1** and **Supplementary Data 1–8**) to a final concentration of 100 ng/μl. The constructs are typically delivered in cloning vectors such as pUC18, pUC19 or pUC57.

2| Prepare the following PCR mix to amplify the designed insert, using a high-fidelity DNA polymerase (NEB Phusion HF or similar) and a primer pair recommended for the DNA vector in which the insert is delivered:

Component	Amount (μl)
Ultrapure water	58
Template DNA (100 ng/μl)	1
GC buffer (5×)	20
Formamide (20% (vol/vol))	10
dNTP mix (10 mM)	2
Fwd primer (10 μM)	4
Rev primer (10 μM)	4
Phusion HF polymerase (NEB)	1
Total	100

▲ CRITICAL STEP For amplification from pUC18, pUC19 or pUC57 plasmids, we use the primers pUC57 fwd and pCU57 rev (**Table 1**).

3| Amplify the designed insert using the following PCR program:

Cycle number	Denature (98 °C)	Anneal (54 °C)	Extend (72 °C)
1	30 s		
2–26	10 s	30 s	40 s
27			10 min

▲ CRITICAL STEP If using a different primer pair, determine the optimal annealing temperature using an online T_m (melting temperature) calculator supported by the polymerase manufacturer. Reduce calculated annealing temperature by 0.6 °C per percentage point of final formamide concentration. Elongation time depends on utilized DNA polymerase and on insert length. For Phusion HF polymerase, use 20 s per kb of insert.

4| After thermal cycling, analyze 5 μl of PCR product by gel electrophoresis on a 1% (wt/vol) agarose gel to ensure that a single band of desired size is present.
? TROUBLESHOOTING

TABLE 1 | Primer pair sequences for subcloning PCR and colony PCR.

Purpose	Template plasmid(s)	Primer pair	Primer sequence 5′–3′	Annealing temp. (°C)
Subcloning PCR (Steps 1–3)	pUC57, pUC19 or pUC18	pUC57 fwd	GTAAAACGACGGCCAGTG	54
		pUC57 rev	GGAAACAGCTATGACCATG	
Colony PCR (Steps 11–13)	pCEP4	pCEP4 fwd	GAGGTCTATATAAGCAGAGC	51
		pCEP4 rev	GCTTATAATGGTTACAAATAAAGC	

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5| Add 10–20 units of DpnI restriction enzyme directly to the amplified PCR product, mix and incubate at 37 °C for 1 h (can also be performed overnight). This step removes template DNA via enzymatic digestion of bacteria-derived methylated GATC sites. Purify PCR products by spin-column purification (Qiagen QIAquick or similar) with final elution in ultrapure water. Determine the DNA concentration using a UV–visible spectrophotometer.

6| Digest 2 μg of purified PCR product with the desired restriction enzymes. This can be performed as a simultaneous double digestion or as sequential digestions with each enzyme, depending on digestion buffer compatibility (follow manufacturers' instructions). Use ten units of each enzyme per μg of DNA and digest for 2 h at 37 °C (proceed with Step 7 while digesting the PCR product). Purify digested inserts by spin-column purification (e.g., Qiagen QIAquick), elute in ultrapure water and determine DNA concentration using a UV–visible spectrophotometer.
▲ **CRITICAL STEP** We typically design antibody constructs with a 5′ KpnI restriction site and a 3′ BamHI restriction site (**Supplementary Data 2–8**), and perform simultaneous digestion of PCR products with KpnI-HF and BamHI-HF restriction enzymes (NEB).

7| At the same time, digest 10 μg of desired mammalian expression vector, using the same enzyme pair utilized for inserts. (Optional) Digest with an additional restriction enzyme that has a restriction site located between the sites of the cloning enzyme pair. This will reduce the likelihood of vector religation in Step 9. Use ten units of each enzyme per μg of DNA and digest for 2 h at 37 °C.
▲ **CRITICAL STEP** We typically subclone designed inserts into pCEP4 expression vector simultaneously digested with KpnI-HF, XhoI and BamHI-HF restriction enzymes (NEB). For subcloning into alternative expression vectors, the choice of restriction enzymes and, consequently, insert design may need to be modified.

8| After vector digestion, add 20 units of calf intestinal alkaline phosphatase, mix and incubate for 1 h at 37 °C. Purify the dephosphorylated vector by spin-column purification (Qiagen QIAquick), elute in ultrapure water and determine the DNA concentration.
■ **PAUSE POINT** The digested vector and inserts can be stored at −20 °C for several years.

9| Ligate the digested insert (Step 6) and digested dephosphorylated vector (Step 8) at a 3:1 insert/vector molar ratio. Mix 100 ng of digested vector, the appropriate amount of digested insert, 1 μl of T4 ligase and 1 μl of 10× T4 ligase buffer in a total volume of 10 μl. Incubate the mixture for 2 h at room temperature.

10| For transformation, add 2 μl of ligation reaction to 20 μl of chemically competent *E. coli* DH5-alpha cells (NEB 5-alpha or similar) and mix. Incubate the cells on ice for 30 min, heat-shock at 42 °C for 45 s and incubate on ice again for 5 min. Add 200 μl of LB medium to the transformed cells and rescue for 1 h at 37 °C, 250 r.p.m. Plate the transformed cells on LB agar plates containing ampicillin (or the appropriate antibiotic for plasmid selection) and incubate overnight at 37 °C.

11| The next day, prepare the following PCR mix (volumes are for a single PCR) using Taq DNA polymerase (Promega GoTaq or similar) and a primer pair recommended for the mammalian vector into which the insert was cloned.

Component	Amount (μl)
Ultrapure water	29.8
GoTaq buffer (5×)	10
Formamide (20% (vol/vol))	5
dNTP mix (10 mM)	1
Fwd primer (10 μM)	2
Rev primer (10 μM)	2
GoTaq polymerase (Promega)	0.25
Total	50

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▲ CRITICAL STEP For amplification from pCEP4 plasmids, we use the primers pCEP4 fwd and pCEP4 rev (**Table 1**).
▲ CRITICAL STEP Always use a Taq DNA polymerase (Promega GoTaq or similar) for colony PCR applications. Avoid using high-fidelity DNA polymerases, as they are readily inhibited by bacterial cell lysates.

12| Mark and number the location of six to eight colonies on the plate. Pick a portion of a colony with a sterile pipette tip, transfer it to the PCR reaction mix and pipette up and down. Do not discard the plates containing transformants. Seal the plates with Parafilm M and store at 4 °C
■ PAUSE POINT The sealed plates can be stored at 4 °C for up to a week.

13| Run the following PCR program:

Cycle number	Denature (95 °C)	Anneal (51 °C)	Extend (72 °C)
1	6 min		
2–36	30 s	30 s	2 min
37			10 min

If you are using a primer pair other than pCEP4 fwd/pCEP4 rev, determine the optimal annealing temperature using an online T_m calculator supported by the polymerase manufacturer. Reduce the calculated annealing temperature by 0.6 °C per percentage point of the final formamide concentration. Elongation time depends on the utilized DNA polymerase and on the insert length. For GoTaq DNA polymerase (Promega), use 1 min per kb of insert.

14| Analyze 5 µl of the colony PCR products by gel electrophoresis on a 1% (wt/vol) agarose gel to verify the presence of the cloned insert. Purify PCR products containing the desired DNA band by spin-column purification (Qiagen QIAquick) and validate the sequence of the insert through Sanger sequencing (you can use the same forward or reverse primer utilized for PCR amplification).
? TROUBLESHOOTING
■ PAUSE POINT Depending on the provider, the sequencing results are typically available between 2 and 3 d after submission.

15| After validation by sequencing, pick colonies containing the correct inserts from stored plates (Step 12) and use them to inoculate 2-ml cultures of LB medium containing ampicillin (or the appropriate antibiotic). Incubate the inoculated tubes at 37 °C, 250 r.p.m. overnight.

16| The following day, make glycerol stocks of selected clones by mixing 700 µl of culture with 300 µl of 50% (vol/vol) glycerol. Place the mixture into a cryotube, snap-freeze it in liquid nitrogen and store it at −80 °C.
■ PAUSE POINT Glycerol stocks can be stored at −80 °C for several years and can be used to inoculate starter cultures for plasmid DNA purification (Steps 17 and 18). To generate a starter culture, stab the frozen glycerol stock with a sterile pipette tip and transfer the tip to a 14-ml round-bottom tube containing 1 ml of LB medium + ampicillin. Incubate the inoculated tubes at 37 °C for 8 h, 250 r.p.m.

17| Use 150 µl of the remaining culture (or starter culture) to inoculate 150 ml of LB medium + ampicillin. Incubate the cultures at 37 °C, 250 r.p.m. overnight.

18| Pellet the bacterial cells by centrifugation at 3,200*g* for 25 min, 4 °C. Discard the supernatants and purify the plasmid DNA from the cell pellets using a plasmid purification kit that contains endotoxin removal steps (e.g., ZymoPURE Maxiprep or similar). Determine the DNA concentration using a UV–visible spectrophotometer. Sterilize the purified plasmid DNA in a laminar flow hood by filtering through a 0.22-µm syringe-driven filter.
? TROUBLESHOOTING
■ PAUSE POINT Sterile DNA preparations can be stored at −20 °C indefinitely and thawed before transfection of mammalian cells.

Expression by secretion into mammalian culture medium ● TIMING 6 d

▲ CRITICAL All steps in this section should be performed in a laminar flow hood, providing a sterile environment

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19| Thaw 1 × 10^7 Expi293F cells in 20 ml of Expi293 medium prewarmed to 37 °C. Spin at 350*g* for 5 min at room temperature and remove the supernatant. Resuspend the cells in 30 ml of Expi293 medium and transfer them to a 125-ml sterile polycarbonate Erlenmeyer flask. Culture the cells at 37 °C, in a 5–8% CO_2 humidified atmosphere with shaking (125 r.p.m.).
▲ **CRITICAL STEP** Allow the cells to reach ~3 × 10^6 cells/ml before first passage (usually 4–5 d).

20| Passage the cells every 3–4 d. When passaging, count the cells and determine their viability using a hemocytometer and trypan blue exclusion. Alternatively, use an automated cell counter. Seed new flasks at 3 × 10^5 cells/ml by diluting the cells in fresh prewarmed Expi293 medium.
▲ **CRITICAL STEP** Cell viability should typically exceed 95%. The doubling time for Expi293F cells is ~24 h, and cultures should reach ~ 5 × 10^6 cells/ml 4 d after passaging. Slower growth rates and lower cell viability may be observed in the first 1–2 passages after thawing.
? TROUBLESHOOTING

21| For cryopreservation of Expi293F cells, resuspend low-passage cultures in Expi293 medium containing 10% (vol/vol) DMSO and store them in sterile cryotubes (1 × 10^7 cells/tube). Place the tubes into a cell-freezing container and freeze at −80 °C. The next day, transfer the tubes to a liquid nitrogen tank for long-term storage.
■ **PAUSE POINT** Expi293 cells can be stored in liquid nitrogen for several years.

22| 24 h before transfection, count the cells and adjust cell density to 2 × 10^6 cells/ml using Expi293 medium. For a 30-ml transfection, a minimum of 40 × 10^6 total cells in culture is recommended.

23| On the day of transfection, count the cells and determine viability. For a 30-ml transfection, add 75 × 10^6 cells to a new 125-ml flask and adjust the volume to 25.5 ml using Expi293 medium. For custom transfection volumes, simply adjust the amount of cells, media, plasmid DNA, transfection reagent and enhancers proportionately, as described in **Table 2**.
▲ **CRITICAL STEP** We recommend transfecting Expi293F cells between passages 3 and 15. Cells may be used up to passage 40; however, researchers should monitor high-passage cultures to ensure that doubling times and viability are not compromised.

24| Prepare DNA for transfection by mixing a total of 30 μg of plasmid(s) from Step 18 with 1.5 ml of Opti-MEM I serum-free medium in a 15-ml tube. Leave the mixture at room temperature until the transfection reagent is ready to use (Step 25).
▲ **CRITICAL STEP** The total amount of transfected DNA should be kept at 30 μg, even if co-transfection of two plasmids is required (e.g., antibody light and heavy chains, **Fig. 5**).
▲ **CRITICAL STEP** For expression of monovalent Fc-fusion proteins, co-transfect Expi293F cells with DNA constructs coding for Fc-fusion and His-tagged Fc (see Experimental design and **Supplementary Data 6** and **7**).
? TROUBLESHOOTING

TABLE 2 | Recommended transfection and culture conditions for the Expi293 transient mammalian expression system[17].

Transfection volume	0.7 ml	1 ml	5 ml	30 ml	100 ml
Plate or Erlenmeyer flask	96-Well (deep well)	24-Well	125 ml	125 ml	500 ml
No. of Expi293F cells in Expi293 medium	1.75 × 10^6 in 0.6 ml	2.5 × 10^6 in 0.85 ml	12.5 ×10^6 in 4.25 ml	75 × 10^6 in 25.5 ml	250 × 10^6 in 85 ml
Plasmid DNA (μg)/ Opti-MEM I (μl)	0.7/35	1/50	5/250	30/1,500	100/5,000
Transfection reagent (μl)/OptiMEM (μl)	1.9/35	2.7/50	13.5/250	81/1,500	270/5,000
Enhancer I (μl)	3.5	5	25	150	500
Enhancer II (μl)	35	50	250	1,500	5,000
Shaker speed (r.p.m.)	1,000 (plate shaker)	190	125	125	125

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25| In a separate 15-ml tube, mix 81 µl of cationic lipid-based transfection reagent (ExpiFectamine 293 or similar) with 1.5 ml of Opti-MEM I and incubate the solution for 5 min at room temperature.
? TROUBLESHOOTING

26| Prepare DNA–lipid complexes by mixing diluted DNA (from Step 24) with diluted transfection reagent (from Step 25), followed by 20 min of incubation at room temperature.

27| Add all 3 ml of DNA–lipid complex mix to Expi293F cells (from Step 23) by dripping the solution while agitating the culture flask. Culture the cells at 37 °C, in a 5–8% CO_2 humidified atmosphere with shaking (125 r.p.m.).

28| The next day, at 16–20 h post transfection, add 150 µl of Enhancer I and 1.5 ml of Enhancer II solutions (from the ExpiFectamine 293 Transfection Kit) to the transfected cells.
▲ CRITICAL STEP Enhancer solutions contain histone deacetylase inhibitors such as valproic acid and sodium propionate[42]. These compounds promote transcriptional activation and have been shown to enhance recombinant protein expression yields in mammalian cells[37,43,44].

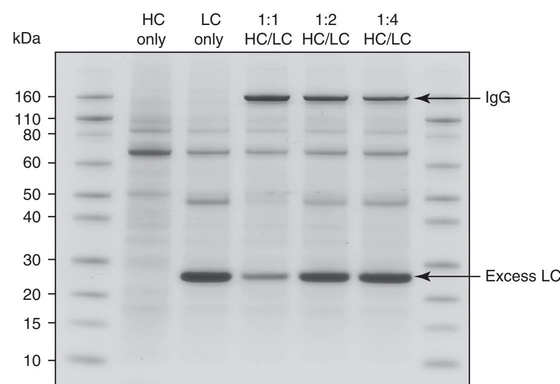

Figure 5 | Optimization of heavy/light chain DNA transfection ratios (IgG format). Expi293F cells (5 ml) were transfected using 5 µg of heavy chain (HC) only, light chain (LC) only or varying ratios of HC/LC DNA, all in the pCEP4 mammalian expression vector. Four days after transfection, 19.5 µl of culture supernatants were analyzed by nonreducing SDS-PAGE. Image densitometry analysis (ImageJ, NIH) revealed that the 1:1 HC/LC transfection ratio resulted in the highest yield of fully assembled IgG, with 1:2 and 1:4 HC/LC ratios resulting in 17% and 32% less IgG, respectively. Transfection of the HC construct alone resulted in no secreted product[50].

29| Harvest the Expi293F supernatant 6–7 d after transfection. Pellet the cells by centrifugation at 3,200*g* for 20 min at 4 °C and filter the supernatants using a 0.22-µm filter unit.
? TROUBLESHOOTING
■ PAUSE POINT The filtered supernatants can be stored at 4 °C for up to 3 d or at −20 °C for several weeks. This, however, may lead to reduced purification yields due to protein aggregation. Refiltering of stored supernatants after thawing is recommended.

Purification of antibody reagents ● TIMING 2 d

30| Purify the expressed antibody-derived molecules by affinity chromatography using immobilized protein A (binds to Fc region and V_H3 domains), protein G (binds to Fc region) or protein L (binds to V_L kappa domains)[45]. Protein A/G/L affinity chromatography (option A) provides a straightforward approach that generally results in high levels of purity after a single purification step. If protein A/G/L affinity chromatography is not feasible, incorporation of a hexahistidine tag (6×HIS) (**Box 1**) for protein purification by means of immobilized metal affinity chromatography (option B) is recommended. The steps below describe the purification of recombinant proteins from a 30-ml transfection volume and aim to minimize contamination with endotoxin. For purification from different volumes of culture supernatant, adjust the affinity resin volume according to the expected protein yield and resin-binding capacity.

(A) Purification of antibody reagents by protein A/G/L affinity chromatography ● TIMING 3 h

(i) Transfer 1 ml of Protein G, Protein L or Protein A pure resin to a new disposable plastic gravity flow column. Wash the resin with 10 column volumes (CVs) of ultrapure water, then equilibrate with 10 CVs of low-endotoxin PBS buffer.

(ii) Apply the filtered supernatant from Step 29 to the column and allow it to flow through by gravity. Wash the resin twice with 10 CVs of low-endotoxin PBS. Collect the flow-throughs from both the supernatant and the washes, and keep them on ice or at 4 °C before SDS-PAGE analysis (Step 31).

(iii) For elution of bound recombinant protein, add 4 ml of glycine–HCl elution buffer (pH 2.7) and collect the flow-through into a pyrogen-free tube prefilled with 1 ml of Tris-HCl solution (pH 7.6). Collect additional fractions as above. Stop elution when the $OD_{280\ nm}$ of the fractions is zero (this is usually after three to five fractions).
■ PAUSE POINT Elution fractions can be stored at 4 °C for up to 3 d. This, however, may lead to reduced purification yields due to protein aggregation.

(iv) After elution, subject the resin to sequential washes with 10 CVs of glycine–HCl (pH 2.7), 10 CVs of low-endotoxin PBS, 10 CVs of ultrapure water and 10 CVs of 20% (vol/vol) ethanol. Store the resin in 20% (vol/vol) ethanol at 4 °C for future use.

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(B) Purification of antibody reagents by immobilized metal affinity chromatography ● TIMING 3 h

(i) Dispense 1 ml of TALON metal affinity resin into a 15-ml pyrogen-free tube. Top up with low-endotoxin PBS buffer, mix the solution and centrifuge it at 300*g* for 2 min at room temperature. Discard the supernatant and repeat the wash step. Resuspend the washed resin in 5 ml of low-endotoxin PBS buffer.

(ii) Using a pH test strip, ensure that the pH of the filtered supernatant from Step 29 is >pH 6.5. Add the resuspended TALON resin to the filtered supernatant and mix gently at room temperature using a rotating mixer at 20 r.p.m. for 20 min.

? TROUBLESHOOTING

(iii) Transfer the supernatant/resin mix to an unused disposable plastic gravity flow column and collect the flow-through. Wash the column twice with 10 CVs of TALON wash buffer.

? TROUBLESHOOTING

(iv) Elute the bound recombinant protein by adding 3–5 ml of TALON elution buffer and collect the flow-through into a pyrogen-free tube. Repeat the process until the $OD_{280\ nm}$ of the fractions is zero (this is usually after three to five fractions).

▲ CRITICAL STEP If purifying monovalent Fc-fusion proteins (see Experimental design), bind the supernatant to TALON resin and elute with ten 3ml fractions containing increasing concentrations of imidazole (5 mM to 50 mM in 5 mM increments, followed by final elution with 10 ml of TALON elution buffer) to separate monovalent Fc-fusion (one 6×HIS tag) from Fc-His dimers (two 6×HIS tags). Bivalent Fc-fusion (no 6×HIS tag) can be purified from the flow-through using Protein G beads as described in Step 30A.

■ PAUSE POINT Elution fractions can be stored at 4 °C for up to 3 d. This, however, may lead to reduced purification yields because of protein aggregation.

(v) After elution, remove the imidazole from the resin by adding 10 CVs of TALON regeneration buffer, followed by washes with 10 CVs of ultrapure water and 10 CVs of 20% (vol/vol) ethanol. TALON resin can be stored in 20% (vol/vol) ethanol at 4 °C for several months.

▲ CRITICAL STEP To completely regenerate the resin, strip cobalt ions with 10 CVs of 100 mM EDTA (resin turns white), followed by two washes with 10 CVs of ultrapure water. Recharge the resin with 10 CVs of 100 mM cobalt chloride hexahydrate (resin becomes pink again). Wash the resin with ultrapure water and store it in 20% (vol/vol) ethanol.

31| Analyze all fractions by SDS-PAGE. Mix 19.5 µl of each fraction with 7.5 µl of 4× LDS sample buffer and either 3 µl of ultrapure water (nonreduced sample) or 3 µl of 100 mM DTT (reduced sample). Also prepare nonreduced samples for supernatant flow-through and washes. Load the prestained protein standard and samples into wells of a 4–12% (wt/vol) Bis-Tris SDS-PAGE precast gel and perform electrophoresis for 35 min at 200 V in MES-SDS running buffer. After electrophoresis, remove the gel from the cassette and use a Coomassie-based dye (InstantBlue or similar) for staining of protein bands.

? TROUBLESHOOTING

32| Combine the fractions with visible protein bands and dialyze against ≥30 volumes of low-endotoxin PBS buffer for ≥2 h at 4 °C using dialysis tubing (SnakeSkin 10K MWCO, Pierce). Perform an additional dialysis step overnight and a final ≥2 h dialysis step the following day.

33| Concentrate the dialyzed protein using a centrifugal concentrator (≤10 kDa cut-off) prewashed with PBS. If the protein stability is unknown, we recommend a moderate speed of centrifugation during concentration (≤1,300*g* at 4 °C) in order to prevent protein aggregation.

▲ CRITICAL STEP We typically concentrate solutions using sequential 15-min centrifugation steps until a final volume of 3 ml is reached and/or until a concentration of 0.5–1 mg/ml is achieved (see ANTICIPATED RESULTS for expected yields). Stop the concentration immediately if the antibody solution becomes cloudy, which is indicative of aggregation.

34| Filter the concentrated sample through a syringe-driven 0.22-µm filter and calculate the protein concentration using its molar extinction coefficient and $OD_{280\ nm}$ reading. The extinction coefficient can be calculated using a protein sequence analysis tool (e.g., ExPASy ProtParam, http://web.expasy.org/protparam/).

35| Prepare aliquots of the concentrated samples and freeze them at −80 °C for prolonged storage. Refilter and determine the protein concentration after thawing.

■ PAUSE POINT The protein aliquots can be stored at −80 °C for several years.

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(Optional) Removal of endotoxin contamination by ion-exchange chromatography ● TIMING 2 d

▲ CRITICAL The protocol below is effective for removing endotoxin from contaminated samples in which the pI of the purified protein falls between 5 and 9. The method relies on adjusting the pH to a point below the pI of the expressed protein but above that of the contaminating endotoxins (which typically have a low pI)[46]. Under these conditions, endotoxins retain an overall negative charge and can be removed from the sample using a strong anion exchange medium. In most cases, this process can be performed using low-endotoxin PBS buffer (Reagent Setup) with the pH adjusted to the desired level.

36| Sterilize and remove all potential aggregates from the sample by filtration through a syringe-driven 0.22-µm filter.

37| Measure the endotoxin content of the purified protein sample using a detection kit (such as the QCL-1000 Endpoint Chromogenic LAL Assay Kit, Lonza). For accurate determination of endotoxin content, measure multiple dilutions of the sample to ensure that one of them falls within the dynamic range of the assay.

38| After endotoxin quantification (in endotoxin units (EU)), calculate the amount of endotoxin contained in a daily dose of purified protein for any planned animal studies. Determine if this is an acceptable or potentially pyrogenic dose, based on existing guidelines (**Box 2**)[47]. If endotoxin content exceeds the pyrogenic threshold, continue with Steps 39–44 below for endotoxin removal.

39| Determine the theoretical pI of the purified protein using a sequence analysis tool (e.g., ExPASy ProtParam, http://web.expasy.org/protparam/).

40| Adjust the pH of low-endotoxin PBS buffer to a pH that is below, but close to the theoretical pI of the purified protein (e.g., for a protein with pI = 6.4, adjust the PBS pH to 6.3). Dialyze against ≥30 volumes of pH-adjusted PBS for ≥2 h at 4 °C, using dialysis tubing (SnakeSkin 10 K MWCO, Pierce). Perform an additional dialysis step overnight and a final ≥2 h dialysis step the following day.

41| Equilibrate a strong anion exchange column (HiTrap Capto Q 1-ml column or similar) with the same buffer as above. Use the flow rate and equilibration volumes recommended by the manufacturer. This process can be performed using an automated system in which the tubing has been depyrogenated with 1 M NaOH, or manually with a disposable syringe and stopcock.

42| Apply the sample to the column at the recommended flow rate. If desired, monitor the $OD_{280\ nm}$ to ensure that the purified protein is flowing through the column and not binding to it.

43| Retest the endotoxin content of the sample as described in Steps 37 and 38. The cleaning process may need to be repeated multiple times to achieve an acceptable endotoxin content. Repeated runs can be performed using new anion-exchange columns or with columns that have been depyrogenated with 1 M NaOH following previous runs.

44| Once adequate endotoxin removal has been achieved, dialyze the sample against low-endotoxin PBS buffer as described in Step 32. Filter the sample through a syringe-driven 0.22-µm filter and determine the protein concentration before storage and/or administration to animals.

■ PAUSE POINT The protein can be aliquoted and stored at −80 °C for several years.

Testing antigen binding by BLI ● TIMING 2 d

▲ CRITICAL The procedure in this section is effective for determining the binding kinetics of a 1:1 interaction between biotinylated target protein coupled to streptavidin BLI biosensors and soluble antibody expressed and purified in the monovalent Fab format.

45| *Biotinylation of target protein antigen (Steps 45–49)*. Centrifuge a 2-ml 7K MWCO desalting column (ZebaSpin or similar) in a 15-ml centrifuge tube at 1,000*g* for 2 min at 4 °C and discard the flow-through. Add 1 ml of PBS to the column, centrifuge the column at 1,000*g* for 2 min at 4 °C and discard the flow-through. Repeat the PBS wash twice. Transfer the PBS-equilibrated column to a new centrifuge tube.

46| Using PBS, dilute the target protein antigen to a concentration ranging from 0.1 to 2 mg/ml in a volume between 0.2 and 0.7 ml. Add the sample to the PBS-equilibrated desalting column and centrifuge the column at 1,000*g* for 2 min

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at 4 °C. Collect the flow-through containing protein, measure the $OD_{280\ nm}$ and use the extinction coefficient to determine protein concentration.
▲ **CRITICAL STEP** The protein antigen is run through a desalting column equilibrated with PBS to ensure the removal of all free amines from the solution (e.g., traces of Tris-HCl or glycine). Chemicals containing free amine groups will interfere with the reaction between antibody molecules and biotinylation reagent.

47| Determine the number of moles of target protein antigen present in the sample based on the volume, concentration and molecular weight. Calculate the number of moles of biotin required for a 20-fold molar excess, e.g., for 1 nmole of protein, 20 nmoles of biotinylation reagent will be required. Determine the volume of a 1 mM stock of biotinylation reagent that must be added to the protein sample to achieve a 20-fold molar excess of biotinylation reagent (e.g., 20 μl for a 1-nmole protein sample).

48| Reconstitute the biotinylation reagent (e.g., EZ-Link NHS-PEG4-Biotin, Thermo Fisher) at 100 mM in DMSO to make a stock solution. Dilute the reagent to a 1 mM working solution using PBS and add the required volume to the protein sample. Incubate the mixture at room temperature for 2 h.
▲ **CRITICAL STEP** It is possible to store aliquots of biotinylation reagent stock solution in DMSO at −20 °C for several months, if handled properly. Avoid any contact with moisture to prevent hydrolysis and inactivation of the NHS-ester reactive group. The biotinylation reagent must be used immediately once diluted in PBS and should be discarded after use.

49| Equilibrate a second 2-ml 7K MWCO desalting column in PBS as described in Step 45. Add the biotinylation reaction mix to the PBS-equilibrated column and centrifuge the column at 1,000*g* for 2 min at 4 °C to remove excess biotinylation reagent from the sample. Measure $OD_{280\ nm}$ and use the extinction coefficient to determine biotinylated protein concentration.
■ **PAUSE POINT** The biotinylated protein sample in the flow-through can be aliquoted and stored at −80 °C for several years.

50| *Assessment of antibody–antigen binding affinity (Steps 50–57).* Rehydrate streptavidin biosensors (Dip and Read, Pall ForteBio) in a biosensor rack with a 96-well plate containing 200 μl/well of 1% (wt/vol) BSA in PBS for at least 10 min at room temperature.

51| Open a new advanced kinetics protocol in the Blitz Pro software (Pall ForteBio). As a default protocol, set all steps to the tube' option, with the following durations:

Step	Program	Time (s)
1	Baseline	30
2	Loading	300
3	Baseline	120
4	Association	180
5	Dissociation	180

52| To perform a blank run, prepare 250 μl of PBS containing 10 μg/ml of biotinylated protein antigen (from Step 49) in a black 0.5-ml microcentrifuge tube, as well as four other black tubes containing 250 μl of PBS. Place the biosensor in a BLItz reader and run the protocol in Step 51 using the tube containing biotinylated protein for the loading step, and PBS tubes for all other steps.
▲ **CRITICAL STEP** Reuse the same biotinylated protein solution for all subsequent steps.

53| Assess the blank run for sufficient loading of biotinylated protein onto the streptavidin biosensor. It is preferable to achieve a loading signal of at least 1 nm and a clear loading plateau to ensure optimal binding signals for subsequent steps.
▲ **CRITICAL STEP** The concentration of biotinylated protein and the duration of the loading step can be modified until adequate loading is achieved.

54| Once adequate loading of biotinylated protein in a blank run has been achieved, select this run as a reference for all subsequent runs.

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55| Perform subsequent runs with biotinylated protein solution for the loading step, a dilution series of soluble Fab (in PBS) for the association step and PBS only for all other steps.
▲ **CRITICAL STEP** If little is known about the affinity of the antibody–antigen interaction, select a broad range of Fab concentrations (e.g., 4 μM in a four-fold dilutions series to 244 pM).

56| Select runs for kinetics analysis ranging between the highest Fab concentration in which binding has reached the maximum possible signal (Rmax) during association and the lowest concentration that yielded a detectable binding signal.

57| Select the global fit analysis option on the BLItz Pro software for the selected runs to obtain models for association rate constant (k_a), dissociation rate constant (k_d) and equilibrium dissociation rate constant (K_D), a quantitative measure of binding affinity ($K_D = k_d/k_a$).
▲ **CRITICAL STEP** The duration of the association and dissociation steps may need to be modified to obtain optimal curve fits.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step(s)	Problem	Possible reason	Solution
4 and 14	No bands or presence of undesired band(s) after DNA electrophoresis	(i) This is indicative of defective and/or unspecific PCR reactions (ii) In some instances, one or both of the utilized primers may share substantial similarity with the insert, leading to the generation of multiple PCR products	(i) In most cases, determination of the optimal annealing temperature for the primer pair used can solve this problem. Optimal annealing temperature for specific primer pairs can be determined using online T_m calculator tools supported by the manufacturer of the utilized DNA polymerase Addition of PCR enhancers such as formamide (final concentration 1–5%) can improve PCR specificity, especially when amplifying GC-rich regions. Note: Each percentage point of added formamide reduces primer T_m by 0.6 °C (ref. 51). (ii) Align the primer and insert sequences using an appropriate tool (e.g., ClustalW) to determine if this is the case. Design new primers targeting a different part of the vector backbone to fix this problem
18	Low yields of plasmid DNA after application of Midiprep or Maxiprep kit	Typical yields for large-scale DNA purification kits range from 0.2 (Midiprep) to 1 mg (Maxiprep) for high-copy-number plasmids. If plasmid yields are lower, it might be because overnight cultures have not been inoculated from actively growing *E. coli* cells	Inoculate cultures from small starter cultures rather than directly from glycerol stocks. For best results, overnight cultures can be inoculated with fresh *E. coli* colonies transformed with plasmid DNA the day before
20	Expi293F cells grow more slowly than expected or not at all	(i) This may occur if culturing high-passage cells (ii) If the problem persists after thawing fresh cells, it could be due to excessive exposure of Expi293 medium to light	(i) Thaw a fresh vial of Expi293F cells to solve the issue (ii) A newly purchased bottle of Expi293 medium will solve the problem. To prevent this problem from occurring, store Expi293 medium at 4 °C in the dark at all times (e.g., inside a box in a cold room) and work in tissue culture cabinets with the light switched off
24	Protein purification yields are suboptimal	Optimization of co-transfection ratios is required (**Fig. 5**)	When co-transfection of two different plasmids is required (e.g., heavy and light chains for IgG or Fab), a good starting point is to transfect at a 1:1 ratio. However, as different plasmids may have different transfection and translation efficiencies, optimization of co-transfection ratios can be performed to improve recombinant protein yields. As IgG and Fab heavy chains require the presence of light chain for secretion[50], optimization of co-transfection ratios is typically performed with excess light chain. An excess of light chain also ensures that purification

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TABLE 3 | Troubleshooting table (continued).

Step(s)	Problem	Possible reason	Solution
24			strategies that rely on heavy chain binding (Fc for IgG and 6×HIS tag for Fab) result in purification of completely assembled IgG or Fab If optimization of co-transfection ratios is required (**Fig. 5**), we recommend testing 1:1–1:5 heavy/light chain ratios in small 5-ml transfections (total of 5-µg of DNA). 3–6 d after transfection, analyze supernatants by SDS-PAGE. In most cases, bands corresponding to overexpressed IgG, Fab and excess light chain can be easily identified. The relative levels of IgG or Fab expression can then be estimated by quantifying the intensity of protein bands using image analysis software (e.g., ImageJ, NIH). If bands are not easily distinguishable, identify expressed IgG or Fab by western blot and quantify band intensity as above. Alternatively, perform small-scale purifications to quantify protein yields directly
25	Protein purification yields are suboptimal	The transfection reagent/DNA ratio requires optimization	There are many commercially available cationic lipid-based transfection reagents. Most manufacturers recommend transfection reagent/DNA ratios ranging from 1:1 to 6:1 (e.g., for ExpiFectamine, a 2.7:1 ratio is recommended) If optimization of transfection reagent/DNA ratio is required, we recommend testing a range of ratios in small 5-ml transfections, as described above
29	Protein purification yields are suboptimal	The protein expression time requires optimization	In most cases, peak levels of protein expression occur at 6–7 d post transfection[17]. However, some proteins and antibodies can display poor stability and a tendency to aggregate[48]. This effect can be accentuated at temperatures required for cell culture (37 °C), which can lead to an overall loss of protein after several days If protein purification yields are considerably lower than expected on days 6 and 7, we recommend performing small-scale (5 ml) transfections and sampling aliquots of supernatant from days 3 to 7. Filter and store aliquots at 4 °C. Perform SDS-PAGE or western blot analysis to determine the peak of protein expression for specific constructs
30B(ii)	Low levels of 6×HIS tag binding to TALON resin	Suboptimal levels of 6×HIS tag interaction with TALON resin can be evidenced by the presence of overexpressed His-tagged protein in the flow-through (visible by SDS-PAGE)	If this is the case, adjust flow-through pH to 8.0 with 1 M NaOH and repurify
30B(iii)	Chelation of cobalt ions from TALON resin	Chelation of cobalt ions may occur when purifying large volumes of Expi293F supernatants (> 100 ml). This can be evidenced by a loss of the resin's pink colouration, leading to poor purification yields and presence of His-tagged protein in the flow-through.	Dialyze supernatant twice against ≥30 volumes of TALON wash buffer to remove chelating agents and re-purify. Another alternative is to replace the Expi293 medium for with one with a reduced content of peptones, such as Pro293 (Lonza)[29]. It is recommended to compare cell growth rates and pre-purification yields before switching to a different cell culture medium.
31	Low or no expression	Optimization of one or more parameters might be required	Refer to troubleshooting advice above for Steps 24, 25, 29, 30B(ii) and 30B(iii)

(continued)

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TABLE 3 | Troubleshooting table (continued).

Step(s)	Problem	Possible reason	Solution
31	Contaminating protein bands in SDS-PAGE	(i) In some instances, protein bands other than the one predicted for the overexpressed protein can be present. Unanticipated formation of protein dimers through intermolecular disulfide bonds can account for the presence of additional bands (e.g., scFv format) (ii) If additional bands are still present under reducing conditions, this may be indicative of unspecific purification and/or protein degradation	(iii) To determine if this is the case, analyze purified protein preparations by SDS-PAGE under reducing conditions (10 mM DTT) in order to determine if only expected polypeptide chains are present (ii) In this instance we recommend performing a second purification step using size-exclusion chromatography[3]

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● TIMING

Steps 1–18, subcloning and large-scale preparation of DNA constructs: 7 d
(Day 1) Steps 1–8, high-fidelity PCR and insert/vector digestion: 8 h
(Day 2) Steps 9 and 10, ligation of inserts and transformation: 4 h
(Day 3) Steps 11–14, colony PCR (5 h) and validation by sequencing (allow 2–3 d)
(Day 5) Step 15, colony picking: 0.5 h
(Day 6) Steps 16 and 17, inoculation of cultures for plasmid DNA purification: 1 h
(Day 7) Step 18, purification of plasmid DNA: 4 h
Expression by secretion into mammalian culture medium: 6 d
(Day 7) Steps 19–22, (optional) thawing and culture of Expi293 cells: 14 d
(Day 7) Steps 23–27, transfection of Expi293F cells: 2 h
(Day 8) Step 28, addition of enhancers: 0.5 h
(Day 13) Step 29, harvest of supernatants: 1 h
Purification of antibody reagents: 2 d
(Day 13) Step 30A, purification of antibody reagents by protein A/G/L affinity chromatography: 3 h
(Day 13) Step 30B, purification of antibody reagents by immobilized metal affinity chromatography: 3 h
(Day 13) Steps 31 and 32, analysis of sample purity by SDS-PAGE and dialysis: 4 h
(Day 14) Steps 32 and 33, dialysis and concentration of antibody preparations: 4 h
(Days 14 and 15) Steps 34 and 35, removal of endotoxin contamination by ion-exchange chromatography: up to 2 d
(Day 15) Steps 36–38, assessment of endotoxin content: 2 h
Steps 39–44, (optional) removal of endotoxin from protein preparations: 2 d
Steps 45–57, testing antigen binding by BLI: 2 d
(Day 15) Steps 45–49, biotinylation of target protein antigen: 4 h
(Day 16) Steps 50–57, assessment of binding affinity: 8 h

ANTICIPATED RESULTS

Typical results for recombinant protein preparations are shown in **Figure 4**. The samples were purified by protein A/G/L affinity chromatography (IgG and IL-2-Fc) or IMAC (Fab and bispecific scFv). A high degree of sample purity was achieved after a single affinity purification step, as analyzed by SDS-PAGE (**Fig. 4a**). When following the recommended steps for prevention of endotoxin contamination (**Box 2**), the purified protein solutions at concentrations required for *in vivo* injection were typically below our established 10 EU/ml threshold (**Fig. 4b**). Binding of expressed antibodies to their respective antigens can be assessed by BLI (or SPR) in order to confirm functionality, and production of Fab fragments can be used to determine true monovalent affinities (see Experimental design and **Fig. 4c**). Yields of expressed antibody-derived molecules vary considerably depending on variable region sequence[48], utilized signal peptide[49] and expressed antibody format. Expression and purification conditions are also important factors that influence recombinant protein yield and must be optimized. In our experience, post-purification yields of up to 100 mg/l for IgG, 100 mg/l for Fab, 130 mg/l for Fc-fusions and 35 mg/l for bispecific scFv can be obtained.

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Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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