Expression of high-affinity human antibody fragments in bacteria

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Here we describe protocols for the expression of human antibody fragments in *Escherichia coli*. Antigen-specific clones are identified by soluble fragment ELISA and concentrated by periplasmic preparation. They are then further purified by affinity chromatography. This article provides an overview of expression and purification strategies for human antibody fragments, as well as detailed protocols for the identification of high-affinity binders and for affinity maturation.

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INTRODUCTION

Antibody fragments represent modular building blocks for the development of larger antibody reagents and therapeutics. Increasingly, they are also directly used in therapeutic applications[1]. Unlike human IgG monoclonals, which generally require expression in mammalian cells, human antibody fragments can be expressed in bacteria at high levels. This includes Fab (VH-CH, VL-CL), scFv (VH-VL) and single-domain (VH, VL) fragments.

One of the major challenges of bacterial expression had initially been the reducing nature of the cytoplasm, which inhibits the formation of intradomain disulfide bridges. Consequently, human antibody fragments had to be refolded *in vitro* from inclusion bodies under oxidizing conditions[2]. More recently, this method has largely been superseded by periplasmic expression, which allows the folding of antibody fragments in the oxidizing environment of the periplasm of Gram-negative bacteria (and *E. coli* in particular)[3,4]. Periplasmic expression in *E. coli* relies on the presence of N-terminal leader peptides that direct the recombinant protein toward protein secretion pathways. Commonly used export pathways include the Sec pathway (and the related ompA, phoA and pelB leader peptides)[3,5] and the signal recognition particle (SRP)[6,7] pathways. The periplasmic space of Gram-negative bacteria is rich in chaperones that aid in the production of folded antibody fragments[8,9]. However, aggregation can still occur, driven by high protein concentrations[10]. This is often less of a problem for phage display–derived fragments that have encountered bacterial expression conditions during the selection process. However, it can affect other antibody fragments. For instance, it has been observed that direct reformatting of IgG into scFv is often unsuccessful, whereas its conversion into Fab tends to be more straightforward[11].

Although antibody fragments can be directly expressed from phage display (phagemid) vectors, higher yields can generally be obtained through recloning into T7-based expression vectors (such as the Novagen pET series)[12]. However, expression levels in phagemids are generally high enough for direct screening of culture supernatants. Thus, some proportion of the expressed fragments will leak into the culture medium (particularly after long incubation times), allowing direct detection of antigen binding by soluble fragment ELISA[13]. The expression of human antibody fragments in a folded state in the bacterial periplasm also greatly simplifies protein purification. For this purpose, the outer membrane of *E. coli* can be removed through a fairly gentle treatment with sucrose and magnesium sulfate. This yields a concentrated and relatively pure preparation of human antibody fragments, devoid of contamination with cytoplasmic proteins. For purification of histidine-tagged fragments, immobilized metal ion affinity chromatography can be used[13]. Alternatively, a highly efficient single-step approach relies on the use of affinity purification on superantigen matrices (protein A, protein L). Whereas protein A binds to human VH3 domains (in addition to its interaction with the antibody Fc region), protein L interacts with human kappa VL domains[14,15]. More recently, an additional matrix has become available that specifically binds to CL (kappa) domains, allowing the affinity purification of Fab fragments (KappaSelect, GE Healthcare).

Technologies for label-free measurements of binding affinities

Once purified antibody fragments have been obtained, determination of antibody fragment–antigen affinities is often required. Alternatively, for applications that may not require an exact knowledge of binding constants, relative affinities allow further ranking of clones. For this purpose, optical biosensor devices are an excellent choice and allow the measurement of antigen-binding affinities without relying on radioactive or fluorescent labels. Arguably, the most popular and long-established technique relies on surface plasmon resonance (SPR) effects. As commercialized by the Biacore AB Corporation it uses carboxymethylated dextran immobilized on a gold chip surface[16]. The instrument detects changes in the SPR signal using polarized light. These changes have been shown to be proportional to the change in mass bound to the chip surface[17], thereby allowing the real-time monitoring of association and dissociation of analytes flowing over the chip surface.

In recent years, many other instruments to determine antibody fragment–antigen affinities have been developed, including microcalorimetry (MicroCal, GE Healthcare)[18], resonant acoustic profiling (TTP, Labtech)[19,20] and biolayer interferometry (BLI). The latter technology has been commercialized by ForteBio through the Octet series of instruments[21]. BLI is an optical analytical technique that analyzes the interference pattern of reflected light. It uses two surfaces: a protein-binding surface on the biosensor tip

and an internal reference surface. Binding of analyte increases the optical thickness of the biosensor tip, which results in a wavelength shift. By using this phenomenon, interactions can be measured in real time, thus allowing the monitoring of association and dissociation rates.

Once affinities have been determined and clones ranked, further increases in affinity may be required. Although association constants of antibody fragments generally fall into a relatively narrow range (10^4–10^6 M^{-1} s^{-1}), large differences in dissociation rates (off-rates) can be observed. Consequently, off-rate selections can be used to further increase the affinity of human antibody fragments[22]. However, direct dissociation of phage-displayed antibody fragments from an antigen-coated surface tends to be influenced by avidity effects. This can be circumvented by using a biotinylated monovalent antigen in combination with a non-biotinylated competitor.

Experimental design

In the first part of the procedure, antigen-specific clones are identified by soluble fragment ELISA. For details of how to select such clones by phage display see Lee *et al.*[23]. For soluble ELISA, individual colonies are grown and antibody fragment expression is induced. Antigen binding is detected using a monoclonal antibody specific for the c-Myc tag present on the antibody fragments (day 3). Periplasmic fractions are obtained via an EDTA/sucrose and magnesium sulfate–based extraction method and dialyzed (day 7). Antibody fragments can be further purified by either protein A/L affinity or Ni-NTA chromatography (day 8). After a second dialysis step, protein concentration is estimated and sample purity is analyzed by SDS-PAGE and size-exclusion chromatography (day 10). The affinity of the purified antibody fragments can then be determined by SPR (**Box 1**) or BLI (**Box 2**). High-affinity clones can be further enriched by off-rate selection (**Box 3**).

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Box 1 | Determination of kinetic and equilibrium dissociation constants by SPR

The Biacore biosensor instruments provide a range of coupling chemistries and associated chip surfaces. Protein can be coupled to the sensor chip via expression tags (such as c-Myc or FLAG) by binding to an anti-tag antibody captured on a protein G superantigen chip (protein G binds to the Fc region of IgG, but does not interact with antibody fragments). Alternatively, proteins of interest can be linked to the chip surface either by covalent coupling by amine-linkage to a proprietary CM5 chip or by capture of biotinylated antigen on a streptavidin chip. The exact experimental parameters, such as the amount of coated sample and buffer composition, will differ for each interaction and generally need to be determined empirically. Generally, the amount of protein coupled to the chip surface should be as small as possible to minimize rebinding effects. As a starting point, a surface density of ~300 resonance units of either antibody fragment or antigen can be used for affinity measurements. Between measurements, the chip surface may have to be regenerated. Again, regeneration conditions have to be established empirically: acid, alkali, high salt and detergent can be used. Once suitable conditions have been established, analyte can be flowed over the chip surface. Samples should be diluted across a suitable concentration range (generally 2–3 logs). Typical concentrations for antibody fragment–antigen interactions are in the range of 1–500 nM. In order to reduce noise and the effect of bulk refractive index changes, data from a blank flow cell should be subtracted from each data set. Most antibody fragment interactions can be fitted to a 1:1 Langmuir model. We recommend fitting data globally, simultaneously using the data from all analyte titrations. It is essential to verify that a 1:1 interaction model is suitable for the specific interaction measured. For instance, many scFv preparations contain a large proportion of dimer, which may cause avidity effects upon binding to the antigen-coated chip surface. This can result in biphasic dissociation curves. See the TROUBLESHOOTING section for guidance on some of the problems that can arise.

Box 2 | Determination of kinetic and equilibrium dissociation constants by BLI (Octet)

BLI biosensor instruments allow the analysis of interactions in either a 384-well or 96-well format. The instruments typically require 100–200 µl of analyte. A more limited range of surface chemistries is available compared with the Biacore SPR platform; however, the range is continuing to expand. Unlike SPR, the Forte Biosciences instruments do not require complex fluidics. The most frequently used setup involves disposable streptavidin tips that are dipped into either the analyte or a control solution. Plates harboring the analyte are gently agitated on a rocking platform; unlike SPR, Biacore (the Octet system) does not require any fluidics. Antibody fragments of interest should be prepared by serial dilution. Typical concentrations of fragments range from 1 to 500 nM. After measurements are complete, data are processed using a double reference subtraction method. Thus, the effect of baseline drift is subtracted using a buffer control well and the effect of nonspecific binding is subtracted using a control antigen solution. As for SPR analyses, most interaction can be fitted to a 1:1 model with global fitting routines.

Box 3 | Affinity maturation of human antibody fragments by off-rate selections

Hawkins *et al.*[22] first described the use of off-rate selection, whereby antibodies are selected on the basis of their dissociation from antigen. Ideally, these selections are performed in solution using monovalent biotinylated antigen and antibody fragments displayed on phage. Fragments can either be selected from naive phage-display repertoires, or, alternatively, from focused libraries generated through diversification of antigen binders. Phages and biotinylated antigen are first allowed to reach a binding equilibrium. Typical concentration of biotinylated antigen used is in the 1–100 nM range. Equilibration times of over 2 h may be required when using low antigen concentrations. Next, an excess of unbiotinylated antigen is added as a competitor at a large molar excess (100–1,000-fold). As phages displaying antibody fragments with fast off-rates dissociate from the biotinylated antigen, they become bound to the molar excess of unlabeled antigen. After incubation, phages that remain bound to biotinylated antigen are captured on streptavidin magnetic beads.

The selection process is strongly influenced by incubation times, which can range from minutes to days. Depending on the desired off-rate, incubation times can be calculated ($t_{1/2}$ = 0.693 / k_{off}) and are as follows:

Incubation time ($t_{1/2}$)	k_{off} required to remain 50% bound
15 min	7.7×10^{-4}
30 min	3.9×10^{-4}
1 h	1.9×10^{-4}
2 h	9.6×10^{-5}
3 h	6.4×10^{-5}

1. Pick a single colony of TG1 bacteria from a freshly streaked M9 minimal medium plate, using sterile toothpicks or pipette tips, and place into a 125-ml disposable flask containing 25 ml of 2xTY medium. Grow overnight at 37 °C with shaking at 250 r.p.m.
2. The next day, measure the OD_{600} of overnight cultures. Inoculate 50 ml of 2xTY medium from the overnight cultures (to an OD_{600} of 0.05). The freshly inoculated cultures should be grown in 125-ml disposable flasks shaken at 37 °C, 250 r.p.m. for 2–3 h to an OD_{600} of 0.5–0.7. Keep the flasks on ice.
3. Add 10^{13} phages to 1 ml of MPBS buffer in an Eppendorf tube. Incubate for at least 1 h at room temperature with gentle agitation (blocking step).
4. Add biotinylated antigen to the phage solution (a typical concentration for the first round of selection would be 50 nM; antigen concentration can be lowered in subsequent rounds). Equilibrate at room temperature with gentle agitation for at least 2 h. As a negative control, phages should be equilibrated without antigen.
5. Add nonbiotinylated competitor antigen at a molar excess of 100–1,000-fold. Incubate for 15 min to 3 h (as above) with gentle agitation.
6. Block streptavidin magnetic beads: transfer 50 µl into a 1.5-ml Eppendorf tube and pellet the beads using a magnet (2 min contact time). Remove the supernatant and resuspend the beads in 1 ml of MPBS buffer. Repeat once and incubate in 1 ml of MPBS for 1 h with gentle agitation. Pellet the beads and resuspend them in 50 µl of MPBS.
7. Add 50 µl of blocked streptavidin magnetic beads to selection. Mix by pipetting and equilibrate for 5 min at room temperature with gentle agitation.
8. Wash six times with 1 ml of PBST, then six times with PBS buffer. Resuspend the beads in buffer, mix by pipetting, capture the beads using a magnet and then remove supernatant by pipetting.
9. Elute the phage from the beads complex by adding 50 µl of either trypsin solution or glycine-HCl elution buffer.
10. Add the eluted phage to 5 ml of TG1 cells (step 2) and mix gently by pipetting.
11. Incubate at 37 °C in a water bath for 1 h.
12. Plate cells on TYE agar plates supplemented with 100 µg ml^{-1} ampicillin and 4% (wt/vol) glucose and grow them overnight at 37 °C.
13. The next day, collect the cells by scraping them from the agar plates in 2xTY medium.
14. Prepare glycerol stocks by adding glycerol (20% (wt/vol) final concentration) and aliquotting in screw-cap cryotubes. Tubes can be stored at −80 °C indefinitely.
15. Repeat for additional rounds of selection, if required. Alternatively, single colonies can be tested for antigen binding by soluble fragment ELISA.

MATERIALS

REAGENTS

- Antibody fragments selected from antibody phage libraries (Tomlinson I + J, domain antibody, ETH-2 or similar)
- TYE agar plates (see REAGENT SETUP)
- M9 minimal medium plates (see REAGENT SETUP)
- 2xTY medium (see REAGENT SETUP)
- Ampicillin solution (Sigma, cat. no. A9518; see REAGENT SETUP)
- Glucose solution (see REAGENT SETUP)
- 96-well round-bottom plate (Costar, Corning, cat. no. 3799)
- Antifoam (Sigma, cat. no. A8311)
- Glycerol (Sigma, cat. no. G5516)
- IPTG (Gold Biotechnology, cat. no. I2481C)
- Maxisorp 96-well ELISA plates (Nunc, cat. no. 442404)

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- Streptavidin ELISA plates (High Bind Streptawell, Roche, cat. no. 11 989 685 001; optional)
- PBS, PBST, MPBS buffers (see REAGENT SETUP)
- Tween-20
- Skim milk powder (Marvel or similar)
- Anti–c-Myc monoclonal (clone 9E10) biotinylated (Sigma, cat. no. B7554)
- Anti–c-Myc monoclonal horseradish peroxidase (HRP) conjugate (Aves Lab, cat. no. CMYC-45P-Z)
- ExtrAvidin-HRP conjugate (Sigma, cat. no. E2886)
- 3,3′,5,5′-Tetramethylbenzidine solution (TMB) (BD Biosciences, cat. no. 555214)
- Sulfuric acid (see REAGENT SETUP) ! CAUTION It is corrosive; wear a lab coat, goggles and gloves
- *E. coli* HB2151 strain (Pharmacia Biotech)
- Screw-cap cryotubes (Nunc, cat. no. 377267)
- Periplasmic extraction buffer 1 (see REAGENT SETUP)
- Periplasmic extraction buffer 2 (see REAGENT SETUP)
- Filter units (0.2 µm; Millipore, cat. no. SLGP 033RS)
- Snakeskin dialysis tubing (Pierce, cat. no. 68100)
- rProtein A Sepharose FastFlow (GE Healthcare, cat. no. 17-1279-02)
- rProtein L resin (Genscript, cat. no. L00239)
- Glycine (Sigma, cat. no. 241261)
- Ni-NTA Superflow resin (Qiagen, cat. no. 30210)
- Imidazole (Sigma, cat. no. I2399) ! CAUTION It is corrosive; wear a lab coat, goggles and gloves.
- Ni-NTA loading buffer (see REAGENT SETUP)
- Ni-NTA wash buffer (see REAGENT SETUP) ! CAUTION It is corrosive; wear a lab coat, goggles and gloves.
- Ni-NTA elution buffer (see REAGENT SETUP) ! CAUTION It is corrosive; wear a lab coat, goggles and gloves.
- Amicon Ultra-15 microconcentrators (10 kDa; Millipore, cat. no. UFC901024)
- NuPAGE 4–12% Bis-Tris SDS-PAGE precast gel (Invitrogen, cat. no. NP0336)
- NuPAGE LDS sample buffer (4×; Invitrogen, cat. no. NP0007)
- NuPAGE MES-SDS running buffer (20×; Invitrogen, cat. no. NP0002)
- Instant Blue protein stain (Expedeon, cat. no. ISB1L)
- Spin-X centrifuge tube filters (0.2 µm; Corning, cat. no. 8160)
- *E. coli* TG1 TR strain (Agilent)
- Dynabeads M-280 streptavidin (Invitrogen, cat. no. 112-05D)
- Trypsin solution (Sigma, cat. no. T1426; see REAGENT SETUP)
- Agar
- NaCl
- Bacto-tryptone
- Yeast extract
- Deionized water
- $MgSO_4$
- $CaCl_2$
- VitB1
- Na_2HPO_4
- KH_2PO_4
- NH_4Cl
- KCl
- Tris-HCl
- Trizma base (Sigma-Aldrich)
- Liquid nitrogen
- DTT

EQUIPMENT

- Incubator (37 °C)
- Shaker (37 °C, 250 r.p.m.)
- Shaker (30 °C, 250 r.p.m.)
- Eppendorf and plate centrifuges (Eppendorf, cat. nos. 5810R and 5415R)
- Sorvall centrifuge (Sorvall)
- Glass flasks (2 liter)
- Plastic boxes
- Disposable culture flasks (125 ml; Corning, cat. no. 430421)
- Disposable plastic columns (Bio-Rad, cat. no. 731-1550)
- UV-visible spectrophotometer
- XCell SureLock electrophoresis system (Invitrogen, cat. no. EI0001)
- ELISA plate reader
- HPLC system (AKTA Purifier or similar, GE Healthcare)
- Biacore SPR system (Biacore 2000 or similar, GE Healthcare; optional)
- Biolayer interferometry system (OctetRed or similar, ForteBio; optional)
- Teflon stir bar
- Pipettes

REAGENT SETUP

TYE ampicillin glucose agar plates Dissolve 15 g of agar, 8 g of NaCl, 10 g of bacto-tryptone and 5 g of yeast extract in 800 ml of deionized water. Autoclave the medium. Cool to 50 °C and add 1 ml of ampicillin solution and 200 ml of glucose solution. Pour the medium into plates. Plates can be stored at 4 °C for up to 4 weeks. Plates should be dried in a flow-bench before use.

M9 minimal medium glucose plates Dissolve 15 g of agar in 800 ml of deionized water. Autoclave the medium. Cool to 50 °C and add 200 ml of 5× M9 salts, 10 ml of 20% (wt/vol) glucose, 1 ml of 1 M $MgSO_4$, 100 µl of 1 M $CaCl_2$ and 1 ml of 1 mg ml^{-1} VitB1. For M9 salts (5× solution), add 64 g of Na_2HPO_4, 15 g of KH_2PO_4, 5 g of NH_4Cl and 2.5 g of NaCl to 1 liter of deionized water and autoclave. Pour the medium into plates. Plates can be stored at 4 °C for several months. Plates should be dried in a flow-bench before use.

2xTY medium Dissolve 16 g of bacto-tryptone, 10 g of yeast extract and 5 g of NaCl in 1 liter of deionized water. Autoclave the medium. Cool the medium to room temperature (25 °C) and add antibiotic solutions and glucose solution as required. Medium can be stored at 4 °C for up to 4 weeks.

Ampicillin solution (100 mg ml^{-1}) Dissolve 1 g of ampicillin in 10 ml of deionized water. Filter through a 0.2-µm filter unit. Aliquot in 1 ml portions. Store at −20 °C for up to 1 year. Thawed aliquots should be freshly diluted 100-fold.

IPTG (100 mM) solution Dissolve 1.2 g of IPTG in 50 ml of deionized water. Filter through a 0.2-µm filter unit. Store at −20 °C for up to 1 year. Thawed aliquots should be freshly diluted 1,000-fold.

PBS buffer Dissolve 3.6 g of Na_2HPO_4, 0.2 g of KCl, 0.24 g of KH_2PO_4 and 8 g of NaCl in 1 liter of deionized water. Adjust the pH to 7.4 and autoclave. Buffer can be stored at room temperature for several months.

PBT buffer PBT buffer is PBS buffer supplemented with 0.05% (vol/vol) Tween-20. It can be stored at room temperature for several months.

MPBS buffer MPBS buffer is PBS buffer supplemented with 4% (wt/vol) skim milk powder. Freshly prepare before use and discard any remaining buffer. Do not store this buffer.

Glucose solution (20%) Dissolve 200 g of glucose in 1 liter of deionized water. Filter through a 0.2-µm filter unit. It can be stored at 4 °C for several months.

Sulfuric acid (1 M) Add 10 ml of concentrated sulfuric acid to 187 ml of deionized water. ! CAUTION Add acid to water, not water to acid, mix in a fume hood and stir with a Teflon stir bar; wear nitrile gloves, goggles and a lab coat.

Periplasmic extraction buffer 1 Periplasmic extraction buffer contains 20% (wt/vol) sucrose, 100 mM Tris-HCl and 1 mM EDTA, (pH 8.0). Dissolve 200 g of sucrose in 900 ml of deionized water. Add 100 ml of 1 M Tris-HCl, adjust pH to 8.0, and then add 2 ml of 0.5 M EDTA. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months.

Periplasmic extraction buffer 2 Periplasmic extraction buffer contains 5 mM $MgCl_2$. Add 5 ml of 1 M $MgCl_2$ to 995 ml of deionized water. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months.

Ni-NTA loading buffer Ni-NTA loading buffer contains 50 mM Tris-HCl and 300 mM NaCl (pH 8.0). Dissolve 17.5 g of NaCl in 950 ml of deionized water and add 50 ml of 1 M Tris-HCl; adjust the pH to 8.0. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months.

Ni-NTA wash buffer Ni-NTA wash buffer contains 50 mM Tris-HCl, 300 mM NaCl and 20 mM imidazole (pH 8.0). Dissolve 17.5 g of NaCl and 1.36 g of imidazole in 950 ml of deionized water and add 50 ml of 1 M Tris-HCl; adjust pH to 8.0. Filter through a 0.2-µm filter unit. It an be stored at room temperature for several months. ! CAUTION Handle imidazole with care; use gloves, goggles and a lab coat.

Ni-NTA elution buffer Ni-NTA elution buffer contains 50 mM Tris-HCl, 300 mM NaCl and 200 mM imidazole (pH 8.0). Dissolve 17.5 g of NaCl and 13.6 g of imidazole in 950 ml of deionized water and add 50 ml of 1 M Tris-HCl; adjust pH to 8.0. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months. ! CAUTION Handle imidazole with care; use gloves, goggles and a lab coat.

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TBSC buffer TBSC buffer contains 10 mM Tris (pH 7.4), 137 mM NaCl and 1 mM $CaCl_2$. Dissolve 1.5 g of Trizma base, 8 g of NaCl and 0.15 g of $CaCl_2$ in 1 liter of deionized water. Adjust pH to 7.4. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months.

Trypsin solution (0.1 mg ml^{-1}) Dissolve trypsin at 10 mg ml^{-1} in TBSC buffer. Freeze as stock in 20-µl aliquots in liquid nitrogen. Aliquots can be stored at −20 °C for several months. Before each experiment, dissolve 20 µl of trypsin stock solution in 2 ml of TBSC to prepare working solution.

Glycine-HCl elution buffer Glycine-HCl elution buffer contains 0.1 M glycine-HCl (pH 2.7). Dissolve 7.5 g of glycine in 1 liter of deionized water. Adjust pH to 2.7 using 10 M HCl. Filter through a 0.2-µm filter unit. It can be stored at room temperature for several months. **! CAUTION** HCl is corrosive; wear a lab coat, gloves and goggles.

PROCEDURE

Screening of clones by soluble fragment ELISA ● TIMING 3 d

1| Pick colonies using sterile toothpicks or pipette tips into a 96-well round-bottom plate containing 200 µl of 2xTY medium supplemented with 100 µg ml^{-1} ampicillin and 4% (wt/vol) glucose.

2| Grow clones in plate overnight at 37 °C at 250 r.p.m. in a plastic box. The 96-well plate can be secured inside the box with pieces of foam or with paper towels. The plate lid should be removed; however, the plastic box should be closed. Carefully keep the box horizontal to avoid spills and cross-contamination.

3| From the overnight cultures, inoculate a fresh 96-well round-bottom plate containing 200 µl of 2xTY medium supplemented with 100 µg ml^{-1} ampicillin and 4% (wt/vol) glucose with 5 µl of the overnight culture. Include a control well which is not inoculated with bacteria.

4| In addition, prepare glycerol stocks of the 96-well overnight cultures by adding glycerol (20% (wt/vol) final concentration) to the plate from Step 2. The plate can be stored at −80 °C indefinitely.

5| Shake the freshly inoculated plate (from Step 3) at 37 °C, 250 r.p.m. for 3 h (in a plastic box, as described in Step 2).

6| Spin cells from Step 5 at 3,200*g* in a plate centrifuge for 10 min. Discard the supernatant by quickly inverting the plate.

7| Resuspend the pellets in 200 µl of 2xTY medium supplemented with 100 µg ml^{-1} ampicillin and 1 mM IPTG by gentle agitation. Grow overnight at 30 °C, 250 r.p.m. in a plastic box for 16–24 h.
? TROUBLESHOOTING

8| The next day, spin the plate at 3,200*g* for 10 min in a plate centrifuge and transfer the supernatant to a new 96-well plate and store it at 4 °C.
PAUSE POINT Plates can be stored at 4 °C for 1–2 d.

9| Coat a 96-well Nunc Maxisorp ELISA plate overnight with 50 µl of antigen solution per well. Alternatively, add 50 µl of 1 µg ml^{-1} biotinylated antigen in PBS to each well of a streptavidin ELISA plate and incubate it at room temperature with gentle agitation for 2 h. Next, wash wells twice with PBST and block with 250 µl per well of MPBS at 4 °C overnight.
? TROUBLESHOOTING

10| The next day, wash the wells of the ELISA plate twice with PBST. Transfer 50 µl of supernatant (from Step 8) to the ELISA plate and incubate it at room temperature for 1 h with gentle agitation.

11| Wash the wells three times with PBST and add 50 µl per well of 1:2,000 biotinylated anti–c-Myc monoclonal 9E10 in PBST supplemented with 2% (wt/vol) BSA. Incubate the ELISA plate at room temperature for 1 h with gentle agitation. Wash the wells with 3× PBST, then add 1:1,000 ExtrAvidin-HRP conjugate in PBST supplemented with 2% (wt/vol) BSA. Incubate at room temperature for 1 h with gentle agitation. Alternatively, when you are using biotinylated antigen or coating with protein A/L superantigen, just wash the wells three times with PBST and add 50 µl per well of 1:2,000 anti–c-Myc monoclonal HRP conjugate in PBST supplemented with 2% (wt/vol) BSA. Next, incubate the ELISA plate at room temperature for 1 h with gentle agitation.

12| Wash the wells with 3× PBST, then add 50 µl of room-temperature TMB solution to each well. Wait for a blue color to develop (1–30 min).

13| Stop the reaction with 50 µl of 1 M sulfuric acid. The color will turn to yellow.

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14| Read the ELISA plate at 450 nm (630 nm subtracted as reference) in a UV-visible plate reader. ELISA-positive clones should display readings of at least 0.2 (after subtraction of reading from control well (Step 3)).
? TROUBLESHOOTING

Periplasmic preparation of soluble antibody fragments ● TIMING 4 d

15| Transform DNA isolated from ELISA-positive clones into HB2151 by electroporation or heat-shock transformation[24].

16| Plate on a TYE agar plate supplemented with 100 μg ml^{-1} ampicillin and 4% (wt/vol) glucose and grow overnight at 37 °C.
■ **PAUSE POINT** The plate can be stored at 4 °C for 2–3 d.

17| The next day, pick single colonies from the plate, using sterile toothpicks or pipette tips, into 125-ml disposable flasks containing 25 ml of 2xTY medium supplemented with 100 μg ml^{-1} ampicillin and 4% (wt/vol) glucose. Grow colonies overnight at 37 °C with shaking at 250 r.p.m.

18| The next day, prepare glycerol stocks of the overnight cultures by adding glycerol (20% (wt/vol) final concentration) and aliquotting in screw cap cryotubes. Tubes can be stored at −80 °C indefinitely.

19| Measure the optical density at 600 nm (OD_{600}) of overnight cultures. Inoculate 500 ml of 2xTY medium supplemented with 100 μg ml^{-1} ampicillin and 4% (wt/vol) glucose from the overnight cultures to an OD_{600} of 0.05.

20| Grow the freshly inoculated cultures in a 2-liter glass flask shaken at 37 °C, 250 r.p.m. for 2–3 h to an OD_{600} of 0.5–0.7.
? TROUBLESHOOTING

21| To induce expression, pellet the cells at 3,200*g* for 10 min and resuspend the cells in 500 ml of 2xTY medium supplemented with 100 μg ml^{-1} ampicillin and 1 mM IPTG. Grow at 30 °C, 250 r.p.m. for 3–14 h.
? TROUBLESHOOTING

22| To pellet cells, spin at 3,200*g* for 10 min. Resuspend the pellet in 25 ml of ice-cold periplasmic extraction buffer 1 and incubate it on ice for 30 min.

23| Spin at 10,000*g* for 10 min and remove the supernatant with a pipette (periplasmic fraction).

24| Resuspend the pellet in 25 ml of periplasmic extraction buffer 2 and incubate it on ice for 20 min. Spin at 10,000*g* for 10 min and remove the supernatant with a pipette (osmotic shock fraction).

25| Pool the periplasmic and osmotic shock fractions. Pass the fractions through a 0.2-μm filter unit and dialyze overnight at 4 °C against PBS buffer (if going on to perform protein A/L affinity chromatography) or into Ni-NTA loading buffer (if going on to perform Ni-NTA affinity chromatography).

Purification of human antibody fragments ● TIMING 1 d

26| Many human antibody fragments can be purified by protein A (VH3 domains) or protein L (kappa domains) affinity chromatography. Protein A/L affinity chromatography (option A) provides a straightforward approach with generally high levels of purity after a single purification step. If protein A/L affinity chromatography is not feasible, many antibody fragments can be purified by means of a C-terminal hexahistidine tag (option B). Sample purity is generally not as high as after protein A/L affinity chromatography and subsequent purification steps may be required (such as ion-exchange chromatography[13] or size-exclusion chromatography).

(A) Purification of human antibody fragments by protein A/L affinity chromatography

(i) Transfer 1 ml of protein A or protein L resin into a gravity-flow column. Wash the resin with 10 ml of water, and then equilibrate the column with 10 ml of PBS buffer.
(ii) Apply dialyzed fractions from Step 25 to the column by gravity flow. Wash the resin twice with 10 ml of PBS.
(iii) To elute the bound antibody fragments, add 10 ml of 0.1 M glycine-HCl (pH 2.7). Manually collect 1-ml fractions in Eppendorf tubes prefilled with 200 mM Tris-HCl (pH 8.0). Measure OD_{280} of fractions and continue collecting until the OD drops below 0.05.

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(iv) Regenerate the resin by adding 10 ml of 0.1 M glycine-HCl (pH 2.7) to the column, allowing it to drain by gravity flow. Then repeat with 10 ml of PBS, twice with 10 ml of water and once with 10 ml of 20% (vol/vol) ethanol. Store the resin in 20% (vol/vol) ethanol at 4 °C indefinitely.

(B) Purification of human antibody fragments by Ni-NTA affinity chromatography

(i) Spin 1 ml of Ni-NTA Superflow resin at 300*g* for 5 min. Discard the supernatant and resuspend the resin in 1 ml of Ni-NTA-loading buffer.

(ii) Add resuspended Ni-NTA resin to the dialyzed fractions from Step 25. Mix gently at 4 °C using a rotating mixer at 20 r.p.m. for 60 min.

(iii) Centrifuge the tubes at 300*g* for 5 min. Carefully remove the supernatant and resuspend the pellet in 5 ml of ice-cold Ni-NTA loading buffer.

(iv) Transfer the suspension into a gravity-flow column. Wash the column once with 4 ml of ice-cold Ni-NTA loading buffer.

(v) Wash the resin once with 8 ml of ice-cold Ni-NTA wash buffer.

(vi) To elute the bound antibody fragments, add 0.5 ml of Ni-NTA elution buffer. Manually collect the flow-through in Eppendorf tubes. Repeat at least four times.

(vii) Measure the OD_{280} of fractions and continue collecting until the OD drops below 0.05.

(viii) Wash the resin with 10 ml of PBS, and then wash twice with 10 ml of water and once with 10 ml of 20% (vol/vol) ethanol, allowing it to drain by gravity flow. Store the resin in 20% (vol/vol) ethanol at 4 °C indefinitely.

Analysis of sample purity by SDS-PAGE and size-exclusion chromatography ● TIMING 2 d

27| Pool all fractions with an OD_{280} higher than 0.05, and dialyze them overnight at 4 °C against PBS buffer. Carefully concentrate the dialyzed fractions in a microconcentrator if required.

? TROUBLESHOOTING

28| Estimate the protein concentration by measuring OD_{280}. Protein concentration can be calculated according to the following formula: concentration (mg ml^{-1}) = $OD_{280\ nm}$ × MW / ε. Molecular weight (MW) and extinction coefficient (ε) can be derived from the antibody fragment sequence (see http://expasy.org/ for details).

■ **PAUSE POINT** Protein can be stored at 4 °C for up to 1 week. For long-term storage, protein should be aliquoted into PCR tubes (10–100 µl) and frozen in liquid nitrogen. Store the aliquots at −20 °C.

? TROUBLESHOOTING

29| Analyze sample purity by SDS-PAGE. For this purpose, first add 1 µg of purified antibody fragment in PBS to 2.5 µl of LDS sample buffer. Add DTT to a final concentration of 1 mM and adjust the volume to 10 µl with water. Heat the samples at 95 °C for 5 min in PCR tubes. Centrifuge the samples at 16,000*g* for 2 min.

30| Load the samples on a 4–12% Bis-Tris SDS-PAGE gel and run it for 35 min at 200 V using MES running buffer.

31| Stain the gel with a Coomassie-based stain (e.g., Instant Blue). Destain extensively in water.

32| Analyze sample purity by size-exclusion chromatography. For this purpose, first dilute 50 µg of purified antibody fragment in PBS up to a total volume of 100 µl. Filter through a 0.2-µm centrifuge filter tube.

33| Equilibrate a size-exclusion column in PBS using an HPLC machine (such as the AKTA Purifier). Inject protein samples and monitor elution from the column by measuring the OD_{280}.

? TROUBLESHOOTING

34| Once pure preparations of ELISA-positive antibody fragments have been obtained, their affinities can be determined by SPR (**Box 1**) or alternatively by BLI (**Box 2**). If fragments with higher affinities are required, these can be enriched by off-rate selection (**Box 3**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

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TABLE 1 | Troubleshooting table.

Step	Problem	Solution
7	Expression in 96-well format for soluble fragment ELISA is poor	Expression in 96-well plates is nonoptimal compared with shaking flasks. Extended incubation times (16–24 h) are crucial
9	Optimizing coating conditions for ELISA	The antigen concentration and buffer conditions used for coating Nunc Maxisorp plates must be optimized for each antigen. As a starting point, 50 μg ml^{-1} of antigen in PBS can be used. In general, capture of biotinylated antigen on streptavidin plates is much more efficient and lower concentrations of antigen can be used. As little as 0.1–1 μg ml^{-1} of biotinylated antigen in PBS is usually sufficient for this purpose
14	No positive clones are obtained in soluble fragment ELISA	Repeat ELISA with a suitable control clone and control antigens (such as the Herceptin-derived 4D5 (ref. 25), which binds HER2 antigen, protein A and protein L). Optimize phage display selection conditions (rounds of selection, antigen concentration, blocking conditions)[23]
20	Excess foaming	Antifoam should be added when using baffled glass flasks
21	Influence of induction time on expression yield	Antibody fragments can be purified by periplasmic extraction after as little as 3 h. Longer induction time can further increase yields. In contrast, this is often counterbalanced by leaking of antibody fragments into the culture medium. However, these antibody fragments can be readily recovered from the culture medium by protein A/L affinity chromatography (spin and filter medium through 0.2-μm filter). We do not recommend Ni-NTA purification from culture medium
27	Protein aggregates during purification or storage	Many human antibodies and human antibody fragments have a tendency to aggregate. Storage at lower concentrations and addition of excipients (such as histidine, trehalose) can limit aggregation. Aggregation during expression frequently results in the formation of inclusion bodies, which can be detected by SDS-PAGE of whole bacterial extract. More recently, engineered human antibody fragments with reduced aggregation resistance have been reported[26–28]
	Protein aggregates on concentration	Protein microconcentrators should be used with caution. Depending on the design, very high local concentrations can occur on the diafiltration membrane. Reduce centrifugation times and repeatedly mix by rinsing the walls of the centrifugation device with buffer
28	Low expression	To achieve the highest level of expression in bacteria, the selected antibody fragments can be recloned into a periplasmic expression vector (such as pET12a, Novagen). This can be achieved by either PCR or, alternatively, by gene synthesis and codon optimization. The pET vector series requires the use of *E. coli* B-strains such as BL21, BL21-Gold or C41 (ref. 29). The use of pET12a is compatible with all of the protocols provided herein
	No expression	Expression and purification should be repeated with a suitable control clone (such as the Herceptin-derived 4D5 (ref. 25)). This clone binds both protein A and L superantigens and can be expressed in scFv, Fab, VH and VL format. Some antibody fragments may contain additional amber stops in a CDR. These clones will have low-level expression in TG1/XL1-Blue MRF′, and no expression in HB2151/BL21/C41
	Expression of fusion protein with pIII	For soluble fragment ELISA, a phage display strain can be used (such as TG1 or XL1-Blue MRF′). However, higher expression yields are generally observed when using *E. coli* HB2151. This strain does not suppress the TGA amber stop codon located between the terminal antibody fragment genes and the phage *pIII* gene. In contrast, in TG1 and XL1-Blue MRF′ a proportion (but not all) of the amber codons are suppressed, resulting in expression of fusion protein to phage *pIII*. It is preferable to use nonsuppressor strains for periplasmic preparations and protein purification. To transfer between strains, plasmid preparations can be transformed into competent HB2151 cells

(continued)

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TABLE 1 | Troubleshooting table (continued).

Step	Problem	Solution
33	Poor resolution on gel-filtration	Elution should be monitored by measuring the OD_{280} over at least 1.5 column volumes. Typical flow rates for analytical gel filtration runs are around 0.5 ml min^{-1}. Superdex 75 columns resolve fragments in the 10–50 kDa range, whereas larger proteins should be analyzed on a Superdex 200 column
Box 1	Biphasic dissociation in Biacore	This is often observed for scFv preparations, which tend to contain a large proportion of dimers. Reduce antigen density on Biacore chip and freshly purify scFv on HR75 gel filtration column

● **TIMING**

Screening of clones by soluble fragment ELISA

Steps 1 and 2, pick colonies: 1 d

Steps 3–8, induce expression: 1 d

Steps 9–14, ELISA: 1 d

Periplasmic preparation of soluble antibody fragments

Steps 15 and 16, transform DNA: 1 d

Steps 17 and 18, pick colonies: 1 d

Steps 19–21, induce expression: 1 d

Steps 22–25, periplasmic extraction: 1 d

Purification of human antibody fragments

Step 26A, purify protein by protein A/L affinity chromatography: 1 d

Step 26B, (day 8) purify protein by Ni-NTA affinity chromatography: 1 d

Analysis of sample purity by SDS-PAGE and size-exclusion chromatography

Step 27, dialysis: 1 d

Steps 28–34, estimate protein concentration, SDS-PAGE, size-exclusion chromatography: 1 d

ANTICIPATED RESULTS

Step 29: yields vary considerably depending on variable regions and expression and purification conditions. As a rule of thumb, typical expression yields for well-behaved antibody fragments (such as 4D5 (ref. 25)) are in the range of: scFv/Fab: 1–5 mg per liter; VH: 5–15 mg per liter, VL: 10–40 mg per liter.

Step 32: typical results for antibody preparations are shown in **Figure 1**. The samples were purified by protein A/L affinity chromatography. A high degree of sample purity was achieved by the single affinity purification step (as analyzed by SDS-PAGE).

Step 33: monomer/dimer equilibrium as observed by size-exclusion chromatography (**Fig. 2**). Shown is a typical result for an scFv preparation, which generally contains a considerable proportion of dimer.

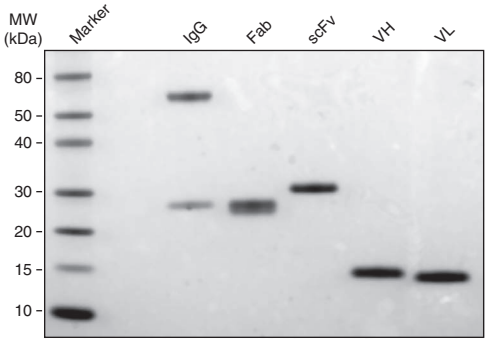

Figure 1 | Analysis of sample purity by SDS-PAGE. One microgram of protein was loaded in each well of a 4–12% (wt/vol)_ Bis-Tris NuPage gel. Expected molecular weights are as follows: IgG, 50 + 24 kDa; Fab, 25 + 24 kDa; scFv, 26 kDa; VH 13 kDa; VL, 12 kDa. Molecular weights can be affected by glycosylation (eukaryotic expression only) and complementarity determining region (CDR) length.

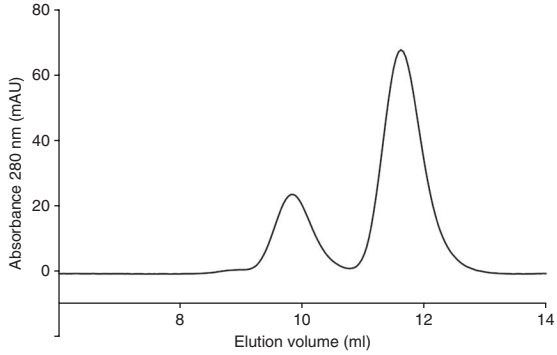

Figure 2 | Analysis of sample purity by size-exclusion chromatography. Fifty micrograms of scFv antibody fragment was loaded onto a Superdex 75 gel filtration column. Expected elution volumes are as follows: monomer, 12 ml; dimer, 10 ml; aggregates, below 9 ml. Protein elution is monitored by measuring absorbance at 280 nm (using an AKTA purifier HPLC system and PBS running buffer at 0.5 ml min^{-1}).

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