203

Recombinant Antibody Expression and Purification

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1. Introduction

1.1. Recombinant Antibodies

Developments in the fields of bacterial expression of functional antibodies and methods to select genes from a library by using the phenotype of the encoded polypeptide have been a breakthrough in antibody technology. Today, phage display in combination with antibody gene libraries is widely used to select *E. coli* host cells that express desired antibody fragments. Such gene libraries are typically produced either from natural sources (e.g., from the spleen of an immunized animal or from plasma cells of human donors) or generated by genetic engineering. The latter has been used to create naïve libraries based on one or more antibody VH and VL gene segments that are diversified by cassette mutagenesis or similar approaches. Such libraries are typically unbiased and can be used for any given antigen ***(1–3)***. Modern naïve libraries are generally large (more than 10^{10} members), contain only few non-functional members, yield antibodies that are well expressed in *E. coli* (more than 1 mg of purified material per liter of culture) and are designed to allow further affinity maturation, if needed. Phage display is then most often used to select desired antibodies from such libraries (*see* ***refs. 4*** and ***5*** for reviews).

Recombinant antibodies offer many advantages over traditionally generated monoclonal antibodies that are only beginning to be explored. These advantages stem from two properties: the ability to assess the antibody DNA within an *E. coli* environment that allows the use of well-known genetic engineering methodologies, and the use of antibody fragments rather than intact IgG molecules, because of their smaller size and because of the absence of the Fc domain. The latter eliminates nonspecific binding to cellular Fc receptors and avoids other, sometimes unwanted, effects caused by the Fc part of intact IgG.

From: *The Protein Protocols Handbook, Third Edition*
Edited by: J.M. Walker © Humana Press, a Part of Springer Science + Business Media, LLC 2009

1.2. E. coli Expression of Recombinant Antibodies

Since antibodies contain disulfide bonds that are necessary for maintaining their overall structure and binding activity, the folding of the molecule has to take place under oxidizing conditions. Gram-negative *E. coli* provides an oxidizing compartment between the cytoplasmic and outer membrane, the so-called periplasmic space. Hence, the attachment of bacterial leader sequences to the N-terminus of an antibody fragment directs the translated antibody chain(s) into the periplasmic space by the bacterial transport apparatus, where folding can take place under oxidizing conditions. To achieve secretion of antibody Fab fragments, both chains must be equipped with signal sequences. In order to cosecrete both chains to the bacterial periplasm, they are best arranged in an artificial operon, thereby being coordinately regulated by one promoter **(Fig. 1)**. In most current systems, the antibody light chain genes precedes the heavy chain gene segment in the two-cistron system, leading to higher expression levels of the light chain. This ensures that the amount of heavy chain without light chain partner is minimized, since unpartnered heavy chain is mostly insoluble, which

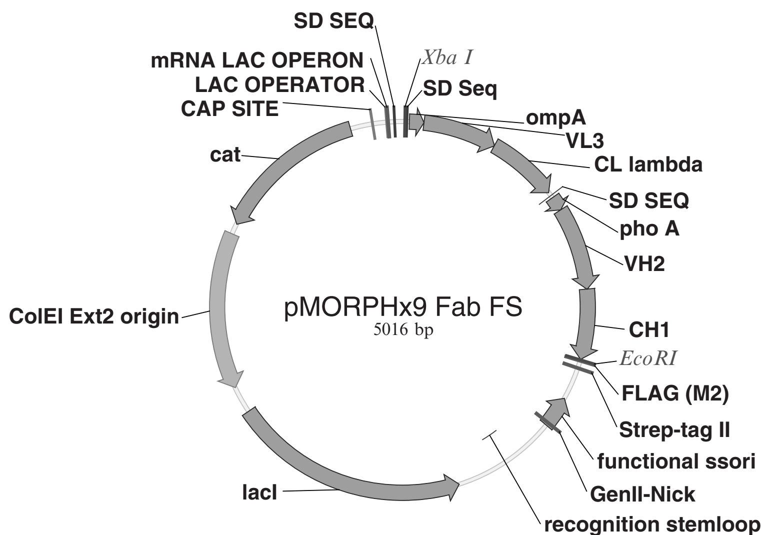

Fig. 1. Schematic view of an antibody Fab expression vector. Features are: a high-copy ColEI origin of replication, a chloramphenicol resistance gene (cat), a lac-promotor/operator region that is inducible by IPTG, and a lacI gene for repression of transcription when the inducer is absent. The Fab gene is arranged as bicistronic unit, flanked by the unique restriction sites XbaI and EcoRI. Each chain is equipped with signal sequences (ompA for the light chain and phoA for the heavy chain). Tags and other functional elements are encoded in frame after the EcoRI site, leading to fusions at the C-terminus of the CH1 domain. In the example shown here, the Fab is fused to a FLAG and and a StrepII tag.

induces a stress to the cell. Since purification tags are fused to the heavy chain (see below), excess light chain will not be purified.

The folding of periplasmic antibody protein does not proceed quantitatively for many antibody fragments. The yield of this process depends on external factors such as temperature, but also on the type of fragment and antibody primary sequence. Modern synthetic antibody libraries use stable and well-expressed antibody frameworks built form codon-optimized sequences, leading to yields of purified active material well above 1 mg per L of standard shaking flask culture **(Fig. 2)**. Such expression yields are sufficient to allow screening of antibody expressing clones after library selection by cultivation in 384 well microtiter plates. After induction of antibody expression, E. coli cells are subsequently lysed (either gently by an osmotic shock procedure to release the periplasmic content, or simply by adding lysozyme). The antibody containing lysates can then be tested by ELISA or other methods for the presence of antigen-specific antibody material. Screening by ELISA is one of the most used and well-established methods to screen antibody expressing clones. Target molecules can be directly coated onto 384-well microtiter plates, or biotinylated target antigens can be captured onto streptavidin plates. Bacterial extracts are added to the micro titer plates and bound antibody fragments are detected with enzyme-conjugated polyclonal antiserum or with antibodies directed against

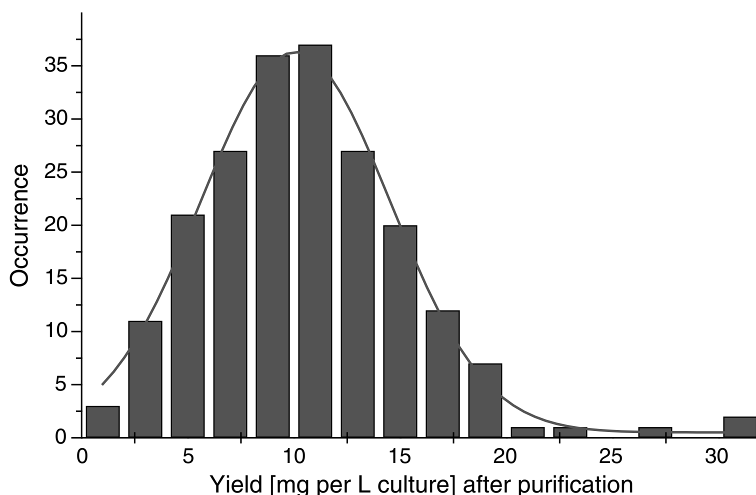

Fig. 2. Histogramm of expression yields after purification from 206 independent clones obtained from the HuCAL® library after phage display selections. Antibodies have been expressed either in monovalent or bivalent Fab format and purified using one-step NiNTA affinity chromatography. Expression yields follow an approximate Gaussian distribution with a center at 10 mg per liter culture volume.

the C-terminal tags provided by the vector (see **Fig. 1**). Often positive hits are sequenced to determine the number of unique antibodies from a selection. The process described above can be automated to a large extent using standard pipetting robots, thereby enabling a massive increase in throughput ***(6)***, one of the advantages over animal-based methodologies. Screening of recombinant antibody fragments on whole cells ***(7)*** and tissues ***(8)*** have also been reported.

After screening, the antibodies can be individually expressed and purified in larger scale. Soluble antibody fragments produced by bacterial colonies isolated as explained above are typically purified by one-step affinity chromatography using peptide tags that have been fused to the C-terminus of the antibody fragment. After purification, the material can be mostly aliquoted and stored frozen, similar to traditional monoclonal antibodies.

1.3. Antibody Formats

The two major types of recombinant antibody fragments that are usually expressed in *E. coli* are named single-chain Fv (scFv) and Fab.

By introducing a short sequence encoding a peptide linker between the VH and the VL gene segments a single polypeptide chain is created that folds into the so-called scFv antibody fragment ***(9)***. A variety of linkers has been tested, and the most frequently used one is a repetition of the sequence Gly_4Ser with 3 or 4 units. It has been shown that shortening the linker between the two variable domains leads to so-called diabodies ***(10)***, which pair with the complementary domains of another scFv and thereby promote the assembly of dimeric or bispecific molecules with two functional antigen binding sites. Since scFv is encoded by a single gene, expression systems do not need coordinated expression of two chains as with the heterodimeric Fab molecule. In addition, in vitro display systems, such as ribosome display ***(11)*** need single polypeptide chains as library members, in order to avoid swapping of chains and thereby loss of the phenotype – genotype linkage.

Another commonly used recombinant antibody fragment is the Fab fragment (**Fig. 3**), which is composed of the truncated heavy chain containing the variable and the first constant region (the so-called Fd fragment), and the entire light chain composed of the variable and constant domain. These two polypeptides are either covalently linked by disulfide bridges at the C-terminus, or are produced in higher yields without those, which nevertheless lead to highly stable H/L heterodimers ***(7)***. Advantages of the Fab fragment are (i), the similarity with the full-length Ig molecule, since it does not contain an artificial linker sequence which might interfere with the antigen binding site, (ii), the fact that well-established anti-Fab detection antibodies can be used and (iii), the fact that it is truly monovalent (which is not always the case with scFv fragments). The latter property is important for example when avidity effects should be avoided, e.g. when the intrinsic binding affinity needs to be determined.

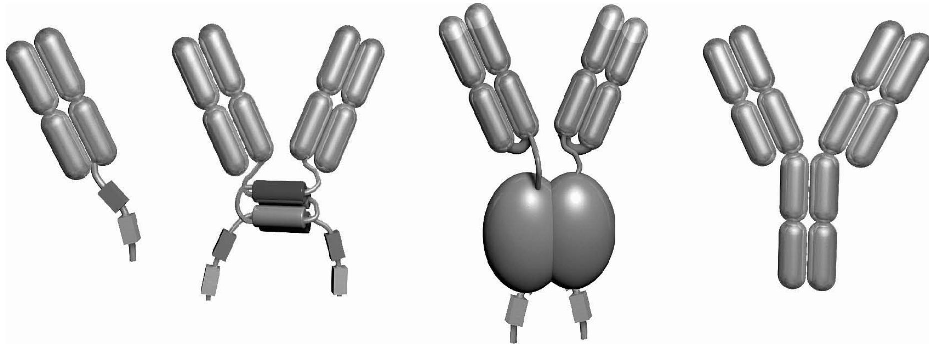

Fig. 3. Comparative schematic view of monovalent Fab with two peptide tags, bivalent Fab (dimerized by a helix-turn-helix motif) with two peptide tags, Fab fused to bacterial alkaline phosphatase (BAP) with one peptide tag, and IgG1. The region involved in antigen binding is shown in golden color. Note that the BAP fusion leads to bivalent Fab molecules, since BAP is a homodimer.

Antibody fragments usually have the same antigen-binding specificity as the corresponding intact antibody, since the complete antigen binding site is present. However, multivalency, which is a very effective means of increasing the functional affinity (avidity) to a surface-bound or polymeric antigen, is a very general property of antibodies. IgG contain two binding sites per molecule, which increases the apparent affinity (avidity) compared to a Fab or monomeric scFv antibody fragment. The most noticeably example is IgM, which carries 10 recognition binding sites. For particular applications, multivalency might be advantageous or even required. Thus, bivalency and further multivalency have been also engineering for recombinant antibody fragments. ScFv or Fab fragments can be dimerized by linking them to a small modular dimerization domain in the form of one or two amphipatic helices ***(12)***. For example, the leucine zipper from the yeast transcription factor GCN4 has been shown to be suitable as a dimerization device. These "miniantibodies" assemble in dimeric form in E. coli with typically no loss in final yield, and the binding performance in assays like IHC or Western blot is mostly indistinguishable from a whole antibody in avidity. Antibody fragments fused to the small tetramerization domain of p53 can form tetrameric molecules ***(13)***.

Genetic fusions to enzymes like bacterial alkaline phosphatase (BAP) connect the binding and detection capability into one molecule (*see* **Fig. 3**). Even such fusions, which lead to molecules of the size of full-length IgG antibodies (about 150 kDa) can be expressed in *E. coli* with almost the same yields as smaller antibody fragments. Since the BAP is a homo-dimer, the resulting molecules are bivalent. Many other antibody fusions for various applications have been described in the literature, such as core streptavidin for avidity increase,

beta-lactamase for prodrug activation or interleukin-8 fusion for neutrophil activation, to name a few. Clearly this field is still in its infancy.

1.4. Peptide Tags for Purification and Detection

Purification of whole antibodies has mostly relied on antigen-affinity chromatography or affinity chromatography using bacterial immunoglobulin-binding proteins such as proteins A and G. However, the usefulness of this strategy for antibody fragments is limited, as these bacterial proteins bind mostly to the constant domains. However, the generation of antibodies *in vitro* enables manipulation of their sequences, for instance by linking desired sequences such as affinity tails to the antibody framework regions. Such affinity tails are often short peptide tags, which are used for purification, but also for antibody immobilization and detection. Typically, such fusions are cloned in-frame at the 3′-end of the antibody gene, leading to a maximum distance in the native fusion protein between the antigen-binding site and the additional functionality. A few such peptide fusions will be highlighted here:

Peptide tags are mostly used for affinity purification purposes. Most tags that have been generally developed for recombinant protein purification will

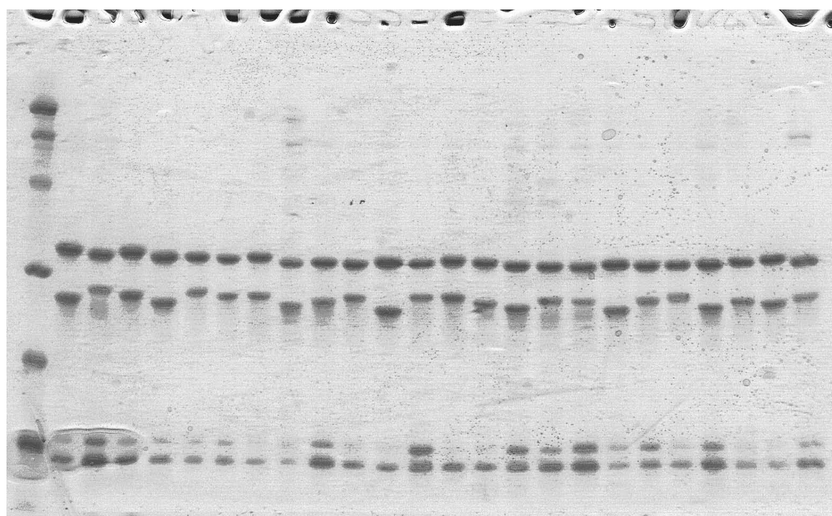

Fig. 4. Coomassie-stained SDS gel (15%, Criterion gel, Bio-Rad, low range marker, Bio-Rad) of 24 different His-tagged Fab antibodies after NiNTA purification, all in the bivalent format. The amount of 1.5 μg total protein was loaded on each lane. The heavy chain (equipped with a helix-turn-helix motif and the myc- and his_6-peptide tags) runs at about 35 kDa, whereas the light chain typically runs below 30 kDa. The impurities at about 15 kDa are C-terminal CH1 fragments that contain the His_6-tag and therefore co-purifiy. They do not contain any aromatic amino acids and therefore do not interfere with A280nm measurements.

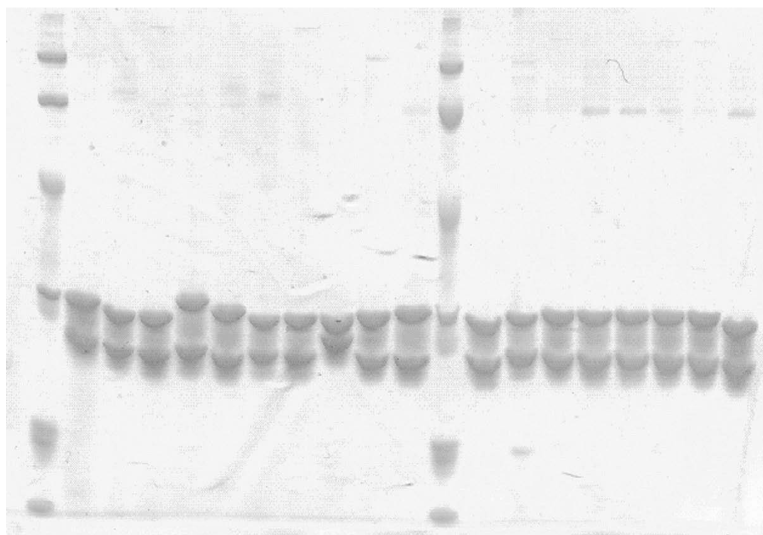

Fig. 5. Coomassie-stained SDS gel (15%, Criterion gel, Bio-Rad, low range marker, Bio-Rad) of 18 different Strep-tagged Fab antibodies after Strep-tag purification. The amount of 3 µg total protein was loaded on each lane. The heavy chain runs at about 31 kDa, whereas the light chain typically runs below 30 kDa. In comparison to the NiNTA purification, the Strep-tag purification combined with the periplasmic preparation will result in significant higher purities (usually >90%).

also work for antibodies. The most convenient strategy is probably the use of a stretch of histidines, the so-called his-tag, a series of 5 to 6 histidines that bind to affinity media such as NTA-agarose or Talon resin, when metal ions (nickel or cobalt) are bound. His-tagged proteins bind with milli-molar affinity to the column and are gently eluted with 150–300 mM imidazole **(Fig. 4)**. Another such tag is the StrepII-tag ***(14)***, which shows affinity to streptavidin- or streptactin-sepharose (streptactin is a genetically engineered streptavidin with higher affinity to the Strep-tag) **(Fig. 5)**. Commercial antibodies to both tags are available, so the tags can be used for detection purposes as well. Other detection tags like the V5-tag (GKPIPNPLLGLDST), which is derived from a small epitope present on the P/V proteins of the paramyxovirus, SV5, or the myc-tag (EQKLISEEDL), which correspond to residues 408–439 of the human p62 c-myc protein are also frequently used, because specific high-affinity monoclonal antibodies are commercially available. The FLAG-tag (DYKDDDDK) is of special interest since a monoclonal antibody (termed M1) exists that only binds to the tag when the N-terminus is free and not involved in a peptide bond. This has been used to monitor cleavage of signal sequences during *E. coli* antibody expression ***(15)***.

2. Materials

2.1. Bacterial Cell Culture

1. 2xYT Medium:
   - 2xYT (Yeast Extract Tryptone) Media; Difco.
   - Dissolve 155 g 2xYT powder in 5 L deionized water.
   - After total dissolution, autoclave at 121°C for 20 min.
   - Store at room temperature.
2. Glucose stock (40%):
   - D(+)-glucose monohydrate, (MW = 198 g/mol).
   - Dissolve 44 g glucose in 100 mL deionized water.
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at room temperature.
3. CAM (Chloramphenicol) stock (34 mg/ml in ethanol):
   - Dissolve 3.4 g chloramphenicol in 100 mL ethanol (p.a.).
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at −20°C.
4. IPTG stock (1M in ddH_2O):
   - Dissolve 4.2 g IPTG in 100 ml deionized water.
   - After total dissolution, sterile filtration using 0.2 µm filter.
   - Store at −20°C.
5. Preculture medium: mix 974 ml 2xYT medium with 25 ml glucose stock solution and 1 ml CAM-stock solution.
6. Expression-culture medium: mix 974 ml 2x YT-medium with 2.5 ml glucose stock solution and 1 ml CAM-stock solution.
7. Sterile filter: Acrodisc 13 mm syringe filters, 0.2 µm (Pall).

2.2. Cell Lysis

1. Cell Lysis Buffer: Take 120 ml BugBuster (Merck), add 240 mg Lysozyme (Roche), 1250 U Benzonase (Merck; 250 U/µl = 5 A/µl), and 5 PIT (Complete Protease Inhibitor) tablets, EDTA free (Roche #1873580) (see **Note 1**).
   Always prepare fresh solution.
2. BSS buffer: 200 mM boric acide, 160 mM NaCl, pH 8.0. Dissolve 12.37 g boric acid add 9.35g NaCl in 1000ml deionized water. Adjust to pH to 8.0 with NaOH. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
3. PeriPrep lysis buffer: 200 mM boric acide, 160 mM NaCl, 2 mM EDTA, protease Inhibitor, pH 8.0. Dissolve 0.75g EDTA: Titriplex®III, (VWR, #108421) and 40 PIT (Complete Protease Inhibitor) tablets, EDTA free, (Roche), in 1000 ml BSS buffer (*see* **Note 1**). Adjust to pH to 8.0 with NaOH.
   Always prepare fresh solution.
4. Avidin stock solution 10mg/ml (for Strep-Tag purifications) (*see* **Note 2**). Dissolve 10 mg avidin in 10ml deionized water. Store at −20°C.
5. Seriflip filter device (STERIFLIP, Milipore).

2.3. Affinity Chromatography

2.3.1. IMAC Affinity Chromatography

1. IMAC-RB (running buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazol, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 0.68g imidazole in 1000ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. IMAC-WB (washing buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazol, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 1.36 g imidazol in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
3. IMAC-EB (elution buffer): 20 mM NaH_2PO_4, 500 mM NaCl, 250 mM Imidazole, pH 7.4. Dissolve 2.4 g NaH_2PO_4, 29.2 g NaCl, 17 g imidazole in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature (*see* **Note 3**).
5. Ni-NTA agarose (Qiagen) (*see* **Note 4**).
4. Gravity flow plastic columns: Poly Prep (BioRad).

2.3.2. Strep Tag Affinity Chromatography

1. Strep-RB (running buffer): 100 mM Tris-HCl, 750 mM NaCl, 1 mM EDTA, pH 8.0. Dissolve 12.1 g Tris, 43.8 NaCl, 0.37g EDTA in 1000 ml deionized water. Adjust to pH to 8.0. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. Strep-EB (elution buffer): Strep-RB + 5 mM D-Desthiobiotin. 100 mM Tris-HCl, 750 mM NaCl, 1 mM EDTA, 5 mM D-desthiobiotin, pH 8.0. Dissolve 12.1 g Tris, 43.8g NaCl, 0.37 g EDTA, 1.1 g D-D-desthiobiotin in 1000 ml deionized water. Adjust to pH to 8.0. After total dissolution, sterile filtration using 0.2 µm filter. Store at 4°C and not longer than 1 week (*see* **Note 5**).
3. Strep-RegB (regeneration buffer): 5 mM HABA, 100 mM Tris-HCl, pH 8.0. Dissolve 1.21 g HABA (HABA-ImmunoPure Pierce/Perbio) and 12.1 g Tris in 1000ml deionized water. Adjust to pH to 8.0 with HCl. After total dissolution, sterile filtration using 0.2 µm filter. Store at 4°C.
4. Streptactin sepharose (IBA-GMBH).
5. Gravity flow plastic columns: Poly prep (BioRad).

2.3.3. Size Exclusion Chromatography

1. PBS: 0.136 M NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, pH 7.4. Dissolve 0.2g KCl, 8g NaCl, 1.44g Na_2HPO_4 and 0.24g KH_2PO_4 in 1000ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.
2. 3xPBS: 0.401 M NaCl, 8.04 mM KCl, 24.3 mM Na_2HPO_4, 4.38 mM KH_2PO_4, pH 7.4. Dissolve 0.6 g KCl, 24 g NaCl, 4.32 g Na_2HPO_4, and 0.72g KH_2PO_4 in 1000 ml deionized water. Adjust to pH to 7.4. After total dissolution, sterile filtration using 0.2 µm filter. Store at room temperature.

3. PD10 columns: GE (Amersham Pharmacia Biotech).

2.3.4. Sterile Filtration, Concentration Determination, SDS-PAGE

1. Sterile filter: Acrodisc 13mm syringe filters, 0.2 µm (Pall).
2. UV-compatible cuvette: UVette (VWR international).
3. SDS-sample buffer 5 x concentrated:
   125 mM Tris-HCl pH6,8 10% glycerol, 0,5% SDS, 0,05% (w/v) bromophenol blue, 5% beta-mercaptoethanol.
4. SDS running buffer: 25 mM Tris, 192 mM glycine, 0,1% SDS.
5. SDS-PAGE system: Criterion 15% Tris. 26W (Bio-Rad).
6. Gel staining solution: Gelcode Blue stain reagent (Pierce).

3. Methods

3.1. Strep-Tag Expression and Purification

3.1.1. Preculture

1. Fill 20 ml medium 1 (2xYT + 1% glucose + CAM) into a 100 ml Erlenmeyer flask.
2. Inoculate culture with a single colony with a sterile inoculating loop. Freshly transformed TG1F$^-$ cells are recommended (*see* **Note 6**).
3. Incubate at 30°C, O/N (18 h +/– 2h), 250 rpm.

3.1.2. Expression Culture

1. Fill 750 ml medium 2 (2xYT + 0.1% glucose + CAM) into a 2 L baffled Erlenmeyer flask.
2. Inoculate with 1/300 volume (2.5 ml) of preculture.
3. Incubate at 30°C, 3 h, 180 rpm.
4. Induce with 560 µL IPTG (1M) stock solution (0.75 mM final concentration).
5. Express Fab at 30°C, 160 rpm, O/N (20 h +/–2 h post induction time) (*see* **Note 7**).

3.1.3. Cell Harvest and Periplasmic Prep (PP)

1. Harvest cells by centrifugation at 5000 g, 30 min, 4°C.
2. Decant supernatant and discard after autoclaving (*see* **Note 8**).
3. Resuspend the bacterial pellet with 30–35 ml cold (<10°C) PeriPrep lysis buffer by pipetting carefully up and down or by shaking at 250 rpm.
4. Transfer suspension into 50 ml Falcon tube and adjust volume to 40 ml.
5. Mix suspension carefully by inverting the tube several times.
6. Incubate O/N, 4°C.

3.1.4. Purification

1. Centrifuge suspension at 16000 g, 4°C, 30 min.
2. Transfer supernatant into a fresh 50 ml Falcon tube by decanting.

3. Add 50 µL avidin stock per sample.
4. Incubate 30 min, 4°C.
5. Filter supernatant with Steriflip filtration device (0.22µm) (*see* **Note 9**).
6. Load a Poly-Prep column with 2 ml Streptactin sepharose (50%; 1 ml bed volume).
7. Equilibrate column with 30 ml Strep-RB by filling once the funnel (~30ml).
8. Load sample to the column and collect the flow through in the respective Falcon tube.
9. Remove funnel and wash twice with 5 ml Strep-RB.
10. Add 0.5 ml Strep-EB and discard flow through.
11. Place column on 2 ml Eppendorf cup.
12. Elute Fab with 2 ml Strep-EB and collect eluate; continue with step buffer exchange.

3.1.5. Regeneration of Strep-Tactin Columns (Optional)

1. After elution regenerate column two times with 5 ml Strep-RegB.
2. Place a funnel onto the column and re-equilibrate the column with 30 ml Strep-RB.
3. Store columns in approx. 2 ml Strep-RB at 4°C (*see* **Note 10**).

3.2. His-Tag Expression and Purification

3.2.1. Preculture

1. Fill 20 ml medium 1 (2×YT + 1% glucose + CAM) into a 100 ml Erlenmeyer flask.
2. Inoculate culture with a single colony with a sterile inoculating loop. Freshly transformed TG1F$^-$ cells are recommended (*see* **Note 6**).
3. Incubate at 30°C, O/N (18 h +/−2 h), 250 rpm.

3.2.2. Expression Culture

1. Fill 750 ml Medium 2 (2 × YT + 0.1% glucose + CAM) into a 2 L baffled Erlenmeyer flask.
2. Inoculate with 1/300 volume (2.5 ml) of preculture.
3. Incubate at 30°C, 3 h, 180 rpm.
4. Induce with 560 µL IPTG (1M) stock solution (0.75 mM final concentration).
5. Express Fab at 30°C, 160 rpm, O/N (20 h +/− 2 h post induction time) (*see* **Note 7**).

3.2.3. Cell Harvest and Whole Cell Lysis (*see* **Note 11**)

1. Harvest cells by centrifugation at 5000 g, 30 min, 4°C.
2. Decant supernatant and discard after autoclaving (*see* **Note 8**).
3. Resuspend the bacterial pellet with 10 ml cold whole-cell lysis buffer by pipetting carefully up and down or by shaking at 250 rpm.
4. Transfer suspension into 50 ml Falcon tube and mix suspension carefully by inverting the tube several times.
5. Incubate for 30 min, at room temperature.
6. Centrifuge suspension at 16000 g, 4°C, 30 min.

7. Transfer the cleared supernatant in a new Falcon tube.
8. Filter supernatant with Steriflip filtration device (0.22 µm) (*see* **Note 9**).

3.2.4. Purification

1. Load a Poly-Prep column with 2 ml Ni-NTA-Sepharose (50%; 1 ml bed volume).
2. Equilibrate column with IMAC-RB by filling once the funnel (~30 ml).
3. Load sample to the column and collect the flow through in the respective Falcon tube.
4. Remove funnel and wash three times with 5 ml IMAC-WB.
5. Add 0.5 ml IMAC-EB and discard flow through (*see* **Note 4**).
6. Place column on 2 ml Eppendorf cup.
7. Elute Fab with 2 ml IMAC-EB and collect eluate; continue with step buffer exchange.

3.3. SEC for Buffer Exchange

3.3.1. Buffer Exchange with PD10 Desalting Column

1. Place column in a rack (e.g. Biorad polycolumn rack) and equilibrate the column with 20 ml PBS (for Fab) or 3xPBS (for bivalent Fab constructs) (*see* **Note 12**).
2. After running buffer through the column, add a total volume of 2 ml of the purified Fab solution.
3. Discard the eluent.
4. When the sample has run into the column, add additional 1 ml of PBS (or 3xPBS).
5. Discard the eluent.
6. When the buffer has run into the column, place a 15 ml Falcon tube under the column and elute the purified Fab with 2.5 ml PBS (or 3xPBS).
7. Wash the column with 20 ml PBS (or 3xPBS).
8. Store columns in approx. 2 ml PBS (or 3xPBS).
   Columns can be reused several times.

3.3.2. Sterile Filtration and Concentration Determination

1. Filter eluate with 0.2 µm filter (Pall; 13 mm) fixed to 2 ml syringe into fresh 15 ml Falcon tube.
2. Take an aliquot of 200 µL for **step 3.3.3**.
3. Fab samples can be stored under sterile condition for several weeks. For long term storage, freezing at −20°C or −80°C is recommended (*see* **Note 13**).

3.3.3. Determination of Concentration and Purity

1. Determine the protein concentration by measuring the absorption at 280 nm (*see* **Note 5**).
   A_{280}nm × 0.65 = concentration in mg/ml (for monovalent Fab constructs).
   A_{280}nm × 0.7 = concentration in mg/ml (for bivalent Fab constructs).
   (dilute sample 1:4 or 1:10 with PBS or (3xPBS) to get values in the linear range between (0.1 and 1AUF).
2. Take up to 5 µg of you protein sample and add with SDS-sample buffer and water to a final volume of 25 µl.

3. Heat sample for 5 min to 95°C for total denaturation.
4. Load protein-sample on 15% Tris-HCl SDS-PAGE (e.g., Biorad Criterion System). Protein amounts between 1.5 and 3 μg are recommended for optimal visual inspection of Fab-bands and impurities.
   Run SDS-PAGE according to manufacture instructions.

4. Notes

1. Use of protease inhibitor mix is not needed in general but recommended to avoid possible degradation of some Fab fragments. The by-products in the purification of bivalent Fab including the BAP fusion construct are not effected by the addition of the protease inhibitor. Be aware that some protease inhibitor mixes contain EDTA or EGTA. Don't use these ingredients in IMAC purification, the metal ion will be chelated and the material will loose binding-activity.
2. Avidin will bind traces of biotin produced by E. coli. In particular, if a Strep-tag column is used several times the biotin will poison the column because it has a much higher affinity to Streptactin as the Strep-tag. At this point washing with Strep-RegB is not sufficient to regenerate the column.
3. Elution of Fab fragments will start at imidazol concentrations of ~50 m*M*, the bivalent construct with two tags will elute at higher concentrations (>75 m*M*).
4. Ni-NTA shows good results in our hands, but some authors prefer other IMAC resins (e.g., Talon-resin or Zn/Cu ions).
5. Desthiobiotin takes a long time to dissolve (gentle warming helps). Low concentrations of desthiobiotin will not interfere with many assays. It also shows no significant UV-absorption or interference in protein concentration assays. The final product is often also very pure (>90% judged on SDS-page). So it is often possible omit buffer exchange and use the eluted product directly in many applications.
6. For optimal expression performance it could be helpful to screen several clones. About 80% of all clones from the HuCAL® library will express in a range of +/−20%. In some case it is possible to find clones which show much higher expression rates (*see* **Fig. 2**).
7. These conditions show the best results for most Fab fragments. Nevertheless it could be helpful to optimise the expression conditions for optimal results. In some cases, lower temperatures (25°C) and lower IPTG concentrations (0.2 mM) will give better results.
8. In some cases (depending on the individual antibody and on the expression conditions, e.g. long expression times, or media that contains glycerol), significant amounts of the Fab can be found in the expression media. This can be checked with an anti-Fab-ELISA.

9. Filtration can be omitted but this may cause clogging or contamination of the columns, in particular if the columns are used several times.
10. Streptactin columns can be used more than 10 times over more than 6 month without significant loss of binding activity.
11. For purification of bivalent Fab or large Fab-fusions it is not recommended to use a periplasmic preparation, because the release in the lysis buffer is often limited and the purification yields drop down.
12. The use of 3xPBS is recommended in purifications of bivalent Fab fragments, because these constructs sometimes tend to aggregate in PBS (depending on the individual antibody and the final concentration). This aggregation can be avoided by using a buffer with higher ionic strength like 3xPBS.
13. After thawing 3xPBS samples, a precipitate can often been seen at the bottom of the well or tube. This is salt from the PBS. It will disappear after thawing and gentle shaking.

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