Chapter 21

Nanobody-Targeted Photodynamic Therapy: Nanobody Production and Purification

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Abstract

Nanobodies have recently been introduced to the field of photodynamic therapy (PDT) as a very promising strategy to target photosensitizers selectively to cancer cells. Nanobodies are known for their characteristic small size (15 kDa), high specificity, and high binding affinities. These features allow rapid accumulation of nanobody-photosensitizer conjugates at the tumor site and rapid clearance of unbound fractions, and thus illumination for activation is possible 1 or 2 h postinjection. Preclinical studies have shown extensive tumor damage after nanobody-targeted PDT. This chapter addresses the first steps toward preparing nanobody-photosensitizer conjugates, which are the nanobody production and purification. The protocol for nanobody production addresses either medium- or large-scale bacterial expression, while the nanobody purification is described for two main strategies: affinity chromatography and ion-exchange chromatography. For the first strategy, protocols are described for different affinity tags and purification from either medium-scale or large-scale productions. For the second strategy, the protocol given is for purification from a large-scale production.

Key words Nanobody production, Nanobody purification, Affinity chromatography, Ion-exchange chromatography

1 Introduction

Nanobodies have recently been introduced to the field of photodynamic therapy (PDT) as a very promising strategy to target photosensitizers selectively to cancer cells [1–4]. Nanobodies are the variable domain of heavy-chain antibodies that exist in animals of the Camelidae family [5] and can also be referred to as VHH (variable domain of the heavy chain of the heavy-chain antibody) or single-domain antibodies. Nanobodies are the smallest naturally occurring antibody fragments, well known for their high specificity and high binding affinities, with potential for distinct applications,

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such as biotechnology, molecular imaging, or cancer therapy [6–8]. In targeted PDT, nanobodies have been shown to efficiently and selectively kill cancer cells overexpressing their target, namely EGFR [1], C-Met [3], and US28 [4]. In preclinical studies, nanobodies have been shown to accumulate rapidly into tumors, to enable rapid clearance of unbound nanobody-photosensitizer conjugates, allowing illumination for activation 1 or 2 h postinjection. Importantly, this strategy has led to extensive tumor damage, with minimal damage to normal surrounding tissues [2].

This chapter addresses the first steps toward preparing nanobody-photosensitizer conjugates, which are the nanobody production and purification. For protocols on earlier steps, such as the preparation of a library for phage display or the selection of nanobodies for a certain target of interest, the reader is referred to other protocols, such as [9–14]. Several nanobody production systems have been described [15] and most of them are based on secretion of the protein into the culture medium, as this simplifies purification substantially. In such cases, nanobodies are provided with a signal sequence that induces transport through the ER-Golgi pathway for eukaryotic production system. The eukaryotic production systems include the yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*, filamentous fungi like *Aspergilli*, or mammalian CHO cells [16–18]. However, these production systems are usually costly and bacterial expression is in most cases sufficient for cheap and rapid production of nanobodies that do not need posttranslational modification for their functionality. Expression of nanobodies in *E. coli* can be in the cytoplasm or by secretion into the periplasmic space by providing the nanobody with an appropriate N-terminal leader sequence, usually a 22-amino acid sequence from PelB. The nanobody production described here concerns medium, i.e., 800 mL, or large, i.e., 5 L, scale production, starting from an already prepared stock of *E. coli* BL21-DE3 transformed with a suitable IPTG-inducible vector for recombinant production in bacteria, containing the sequence of the nanobody of interest, with a pelB sequence for periplasmic localization. Thereafter, two main strategies are described for purification: affinity chromatography and ion-exchange chromatography. The affinity chromatography is described for nanobodies containing different affinity tags, namely histidine-tag (or his-tag) and EPEA-tag (or C-tag), as well as for nanobodies that bind protein A. This strategy is described for purification from either medium-scale or large-scale productions. For the second strategy, i.e., ion-exchange chromatography, the protocol given is for purification from a large-scale production using cation and anion exchange.

2 Materials

2.1 General

1. Luria Bertani (LB): Dissolve 10 g tryptone, 5 g yeast extract, 10 g NaCl in 800 ml Milli-Q and adjust pH to 7.4 with 2 M NaOH. Adjust volume to 1 l with Milli-Q. Sterilize with autoclaving.
2. YT-2x medium: Dissolve 16 g tryptone, 10 g yeast extract, and 5 g NaCl in 800 mL Milli-Q and adjust pH to 7.4 with 2 M NaOH. Adjust volume to 1 L with Milli-Q. Sterilize with autoclaving.
3. Sterile 20% glucose.
4. LB-agar plate, supplemented with appropriate antibiotic and 2% glucose.
5. 1 M Isopropyl β-D-thiogalactopyranoside (IPTG).
6. Appropriate antibiotics and chloramphenicol (30 mg/mL).
7. 1x PBS: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 in Milli-Q; adjust to pH 7.4 with 1 M HCl.
8. SDS-PAGE loading buffer (4x): 200 mM Tris/HCl, pH 6.8, 40% glycerol, 400 mM DTT, 8% w/v SDS, 0.4% bromophenol blue.
9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffers and equipment.
10. NanoDrop Spectrophotometer (Thermo Fisher Scientific).
11. Spectrophotometer (e.g., Eppendorf Biophotometer plus).

2.2 Production with Fermentor (or Bioreactor)

1. Terrific broth (TB), supplemented with 0.089 M potassium phosphate buffer and 0.1% glucose: 60 g tryptone, 120 g yeast extract, 4.5 g glucose, 20 mL glycerol in 4.5 L Milli-Q. Potassium phosphate buffer pH 7.4 contains 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 in 500 mL Milli-Q and is added to TB with a 1:9 ratio. Sterilize both components by autoclaving and add together when cooled down.
2. Antifoam solution.
3. 1 M H_2SO_4.
4. 2 M NaOH.
5. Benchtop fermentor system (or bioreactor) containing a 7.5 L vessel (e.g., Eppendorf BioFlo 115 benchtop fermentor).

2.3 Purification with Beads (Medium Scale)

2.3.1 Purification of Nanobodies Containing Histidine-Tag

1. Beads: Nickel-NTA agarose (QIAGEN).
2. Elution buffer: 300 mM Imidazole; dissolve imidazole in 1x PBS, adjust pH to 7.4 with 1 M HCl.
3. Econo-Pac® Chromatography Column (gravity-flow column, Bio-Rad).

2.3.2 Purification of Nanobodies Containing EPEA-Tag (C-Tag)

1. CaptureSelect™ C-tag Affinity Matrix (Thermo Fisher Scientific).
2. Elution buffer: Tris (20 mM) containing $MgCl_2$ (2 M). First dissolve Tris in demi-water and adjust pH to 7.4. Add $MgCl_2$ and check pH again.

2.4 Purification with ÄKTAxpress (Large Scale)

1. ÄKTAxpress chromatography system with Unicorn software (GE Healthcare), or similar system which has the possibility for multistep protein purification.
2. 2 × 5 mL HiTrap desalting columns, stacked (GE Healthcare).
3. 20% Ethanol.
4. 2 M NaCl.
5. 0.1 M HCl.
6. 0.1 M NaOH.
7. 0.45 μm Bottle-top 500 mL PES filters.

2.4.1 Purification of Nanobodies Containing Histidine-Tag

1. Columns: 1 mL or 5 mL HisTrap FF crude column (GE Healthcare).
2. Binding buffer: 20 mM Sodium phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH 7.4.
3. Elution buffer: 20 mM Sodium phosphate buffer, 0.5 M NaCl, 300 mM imidazole, pH 7.4.

2.4.2 Purification of Nanobodies Containing EPEA-Tag (C-Tag)

1. Columns: 1 mL or 5 mL CaptureSelect™ C-tag column (Thermo Fisher Scientific).
2. Binding buffer: 20 mM Sodium phosphate buffer, 150 mM NaCl, pH 7.4, or PBS.
3. Elution buffer: 20 mM Tris, 2 M $MgCl_2$, pH 7.

2.4.3 Purification of Nanobodies with Affinity for Protein A (see **Note 1**)

1. Columns: 1 mL or 5 mL HiTrap Protein A HP column (GE Healthcare).
2. Binding buffer: 20 mM Sodium phosphate buffer, 150 mM NaCl, pH 7.4, or PBS.
3. Elution buffer: 0.1 M Citrate buffer, pH 3.

2.4.4 Purification of Nanobodies with Cation-Exchange Column

1. Columns: 1 mL or 5 mL HiTrap SP HP column (GE Healthcare).
2. Binding buffer (A): 25 mM Sodium acetate buffer, pH dependent on pI of purified nanobody.
3. Elution buffer (B): 25 mM Sodium acetate, 1 M NaCl, pH dependent on pI of purified nanobody (the same as pH of buffer A).

2.4.5 Purification of Nanobodies with Anion-Exchange Column

1. Column: HiPrep™ Q XL 16/10 column (GE Healthcare)
   Buffer: 1x PBS
2. Elution buffer is not needed as the purified nanobody is in the flow through, whereas impurities bind to the column.

3 Methods

3.1 Medium-Scale Production of Nanobody (800 mL of Culture Media)

1. Streak BL21-DE3 bacteria from the appropriate glycerol stock onto an LB-agar plate using a sterile loop, toothpick, or pipette tip. Incubate the plate O/N at 37 °C.
2. Start an O/N pre-culture by inoculating a single colony from a bacterial plate in LB medium, supplemented with 2% glucose, appropriate antibiotic, and 30 μg/mL chloramphenicol (*see* **Note 2**). Incubate O/N at 37 °C on a shaker.
3. Prepare 800 mL of growth solution for the production of nanobody by mixing YT-2x medium containing 0.1% glucose, appropriate antibiotic, and O/N culture of bacteria (1 mL of O/N culture should be added to 100 mL of growth solution, *see* **Note 3**). Incubate the flask for 3–4 h to reach optical density ($OD_{600\ nm}$) between 0.5 and 0.7. Add IPTG (final concentration of 1 mM) and shake the flask for 16 h at 22–25 °C.
4. Measure the $OD_{600\ nm}$ before harvesting the cells (*see* **Note 4**). Centrifuge the bacterial culture at 6000 × *g* for 20 min at 4 °C. Discard the supernatant and resuspend the pellet in 20 mL of PBS. Freeze/thaw the cells twice to disrupt the outer cell membrane and release the periplasmic content, which contains the nanobody.
5. Centrifuge the cells at 12,000 × *g* for 20 min at 4 °C. Transfer the supernatant to a clean falcon tube. Keep the pellet in freezer for analysis on SDS-PAGE gel. Purification of the nanobody is explained in Subheading 3.3.

3.2 Large-Scale Production of Nanobody (5 L of Culture Media)

The procedure described below is intended for using the Eppendorf BioFlo 115 benchtop fermentor (or bioreactor). At some points in the procedure there is a referral to the manufacturer's manual for more detailed descriptions.

3.2.1 Preparation of Bacteria

1. Follow Subheading 3.1, **step 1**.

3.2.2 Vessel Sterilization and O/N Bacterial Pre-culture

1. Follow Subheading 3.1, **step 2**, to prepare O/N culture.
2. Prepare the 5 L fermentor vessel for sterilization; check whether all necessary parts are in the vessel and install the DO_2 (dissolved oxygen) probe and pH probe according to the manufacturer's manual.

3. Add 2 L demi-water to the vessel and sterilize by autoclaving for 25 min at 121 °C.

3.2.3 Production in Fermentor

1. After sterilization, position the vessel next to the BioFlo 115 control cabinet, and tighten the screws on the head plate.
2. Put the sample kit back in place and tighten the glass bottle (make sure that the metal clip is tight on the silicon tube). Secure all connections.
3. Connect the cooling lines to the cooling loop and the exhaust/condenser and start the watercooler (switch ON and then press the enter button for a few seconds).
4. Turn on the control cabinet.
5. It takes around 2 h for the DO_2 probe to get polarized before it can be calibrated. Calibrate its zero value to "0" and then connect the cable. When the DO_2 probe is polarized set its span value. Set the DO_2 to 70% and Auto.
6. Carefully place the agitation motor on top of the vessel and put the heating blanket around the vessel (approximately 1 cm below media level).
7. Add a small amount of demi-water to the temperature probe holder and put it in until it reaches the bottom.
8. Connect the air supply from the fermentor to the sparger.
9. Connect the pH sensor to its corresponding data cable.
10. Remove the H_2O using the harvest tube and replace with the short silicon tube (close the clip).
11. Add 5 L TB medium (supplemented with potassium phosphate buffer, 0.1% glucose, appropriate antibiotic, and 500 μl antifoam) using a sterile funnel. Put 1 mL sample aside which can be used as "blank" when measuring ODs at 600 nm on a spectrophotometer.
12. Calibrate the pH sensor according to the manufacturer's manual.
13. Insert the tubing of the base and/or acid into the pumps and "prime" them. Use syringe needles to bring acid and base in the vessel via the two septum ports.
14. Set pH to Auto (choose for pH 7.0 ± 0.1) and set the fermentor to the Agit cascade and Agit to Auto (max agit 1000 rpm and min 200) (*see* **Note 5**).
15. Heat up the media to 37 °C. Set Temp to Auto.
16. Connect the foam trap to the exhaust/condenser.
17. Allow gas to flow in the vessel (adjust left gas controller up to 1) until the DO_2 probe gives a stable signal.
18. Start up the BioCommand® supervisory software to monitor all parameters during the fermentor production.

19. As soon as all parameters have reached the optimal value (DO_2 will be higher than 70%), start the inoculation by adding 50 mL (1:100 dilution) of the overnight culture using the big port on the top.
20. Allow the bacteria to grow at 37 °C until the desired OD is reached. Use the sample kit to take samples to monitor the OD at 600 nm.
21. Once the $OD_{600\ nm}$ is in log phase (usually between 2 and 4; *see* **Note 6**), add IPTG to a final concentration of 1 mM.
22. Lower the temperature for O/N production to 25 °C.

3.2.4 Harvesting Bacterial Culture

1. Take two samples with a 20-min interval to monitor whether the OD is stable. If the OD is stable and not increasing anymore, choose "End batch" in BioCommand (look in Batch Control).
2. Set everything to OFF (including gas) apart from agitation and remove the blanket.
3. Connect the harvest tube and collect the culture.
4. Turn off Agit, prime the pumps out, and switch them off. Switch off the console and the cooler.
5. Clean the fermentor vessel and tubing according to the manufacturer's manual.
6. Centrifuge the culture at 6000 × *g* for 20 min at 4 °C. Resuspend the pellet in PBS (180 mL for 5 L culture) and freeze/thaw two times. The purification is described in Subheadings 3.4 and 3.5.

3.3 Purification of Nanobodies with Affinity Chromatography Approach Using Gravity-Flow Column

This protocol refers to the purification of nanobodies using Nickel (for nanobodies with histidine-tag, his-tag) and CaptureSelect™ C-tag Affinity Matrix (for nanobodies with EPEA-tag or C-tag) with gravity-flow column, and thus from medium-scale nanobody production.

1. Add appropriate amount of beads to a small column and wash with PBS to remove storage buffer (*see* **Note 7**).
2. Load the periplasmic fraction on the column and incubate for 1 h at 4 °C on a shaker.
3. Collect the flow-through and wash the beads with cold PBS. Repeat this step until the absorbance of flow-through at 280 nm approaches baseline.
4. Elute nanobody containing his-tag with two resin-bed volume of 300 mM imidazole solution. Repeat this step to collect all bound nanobody. To elute a nanobody containing EPEA-tag from C-tag Affinity Matrix, use Tris-$MgCl_2$ elution buffer. Monitor the absorbance of fractions at 280 nm.

5. To remove imidazole or $MgCl_2$ use gel filtration or dialysis.
6. Analyze purified proteins by SDS-PAGE.

3.4 Purification of Nanobodies Through Affinity chromatography with ÄKTAxpress System

The method stated below can be used for nanobodies containing either the histidine-tag or the EPEA-tag or for nanobodies which can be purified using protein A (*see* **Note 8**). The purification steps are similar and differ only in buffer usage. The buffers and columns for each type of affinity purification are indicated under materials. The method includes a second chromatography step using desalting columns to exchange the buffer used in the first chromatography step against PBS.

3.4.1 Sample Preparation and Column Attachment

1. Filter all buffers through a 0.2 μm PES filter and degas until all dissolved air bubbles have disappeared (use a vacuum pump, *see* **Note 9**).
2. Filter the bacterial periplasm using a 0.45 μm vacuum filter and check the pH. Adjust if necessary (*see* **Note 10**).
3. Attach the appropriate affinity column on column position 1 (depending on the nanobody, either HisTrap, CaptureSelect™ C-tag, or protein A HP) and two stacked HiTrap desalting columns on column position 5 of the ÄKTAxpress system (*see* **Note 11**).

3.4.2 Nanobody Purification and Buffer Exchange

The following steps can be included in a single purification program, but will be addressed separately. A purification program can be made beforehand using the “method wizard” function available in the Unicorn software. During purification all parameters (flow rate, pressure, absorbance) should be monitored and intervention is possible at each step.

1. Remove 20% ethanol from the system (including sample loading tubing) and the columns using Milli-Q water (*see* **Note 12)**.
2. Equilibrate the system and columns (including the sample loading tubing) with at least five column volumes (CV) of binding buffer for the affinity columns (position 1). Equilibrate the desalting columns with 5 CV of PBS (position 5).
3. Load the sample (bacterial periplasm) onto the column with a flow rate of 1 mL/min or 5 mL/min, for the 1 mL or 5 mL columns, respectively (*see* **Note 13**).
4. Wash out the sample with 10–15 CV of binding buffer, until the absorbance reaches a steady baseline.
5. Elute the sample with 5 CV 100% elution buffer (one-step block elution). Make sure that the eluted peak fractions will be stored in one of the five sample loops (10 mL) within the system.

6. Wash out any remaining protein with an additional 5–10 CV of elution buffer.
7. Wash the pumps and system with PBS.
8. Load sample from sample loop onto the stacked HiTrap desalting columns with a flow rate of 5 mL/min and collect peak fractions (*see* **Note 14**).
9. Elute the sample with 1.1 CV PBS (11 mL). Make sure that the peak fractions are collected using the fraction collector.
10. Wash system and columns with 5–10 CV of Milli-Q water, followed by 5–10 CV of 20% ethanol.
11. Analyze obtained fractions by SDS-PAGE (*see* **Note 15**).

3.5 Purification of Nanobodies Using Ion-Exchange Chromatography

The method stated below can be used for purification of nanobodies devoid of purification tags, such as the histidine-tag or the EPEA-tag. The ion-exchange chromatography protocol is based on the separation of molecules due to their charge. Depending on the pH of the buffer, nanobodies may carry a net positive charge, a net negative charge, or no charge. The isoelectric point (i.e., pI) is a pH at which a molecule has no net charge. The pI value can be determined experimentally or in silico based on the primary sequence of the molecule, using, e.g., ProtParam Tool. As the choice of buffer pH affects the net charge of the nanobody, when purifying the nanobody using a negatively charged cation-exchange resin the nanobody should carry a positive net charge. In order for the nanobodies to carry a positive net charge a buffer with a pH lower than the pI of the nanobody should be used. The buffers and columns used in this procedure are indicated under materials. The method includes two consecutive chromatography steps, indicated as STEP 1 and STEP 2.

3.5.1 Sample Preparation and Column Attachment

1. Filter all buffers as described in Subheading 3.4, **step 1**.
2. Filter the bacterial periplasm as described in Subheading 3.4, **step 2**. The first purification step involves a negatively charged cation-exchange resin; therefore adjust the pH of the periplasm to 0.5–1.5 pH units less than the pI of the nanobody (*see* **Note 10**).
3. Attach the HiTrap SP HP cation-exchange chromatography column on column position 1.

3.5.2 Nanobody Purification Using the HiTrap SP HP Cation-Exchange Chromatography Column (STEP 1)

A purification program can be made beforehand using the "method wizard" function available in the Unicorn software. During purification all parameters (flow rate, pressure, absorbance) can be monitored and intervention is possible at each step.

1. Remove 20% ethanol from the system as described in Subheading 3.4, **step 4**.

2. Equilibrate the system and columns (including the sample loading tubing) with at least five column volumes (CV) of binding buffer A for cation-exchange column (*see* **Note 16**).
3. Load the sample (bacterial periplasm) onto the column with a flow rate of 1 mL/min or 5 mL/min, for the 1 mL or 5 mL columns, respectively.
4. Wash out the sample with 10–15 CV of binding buffer A, until the absorbance reaches a steady baseline.
5. Elute the sample with increasing gradient set to 100% target buffer B. Make sure that the eluted peak fractions are collected.
6. Wash out any remaining protein with an additional 5–10 CV of elution buffer (100% buffer B).
7. Pull together fractions collected in **steps 8** and **9**.
8. Wash system and columns with 5–10 CV of Milli-Q water, followed by 5–10 CV of 20% ethanol.
9. Analyze obtained fractions by SDS-PAGE.

3.5.3 Nanobody Purification Using the HiPrep™ Q XL 16/10 Column Anion-Exchange Chromatography Column (STEP 2)

1. Attach the HiPrep™ Q XL 16/10 column anion-exchange chromatography column on column position 1.
2. Wash the column in Milli-Q water to remove ethanol.
3. Wash the column in 0.1 M NaOH at a flow rate of 5 mL/min for 4 min.
4. Wash the system in Milli-Q water at a flow rate of 5 mL/min for 10 min.
5. Wash the system in PBS at a flow rate of 5 mL/min for 10 min.
6. Load sample obtained in **step 10**.
7. Collect the flow-through as this fraction contains purified nanobody.
8. Wash the system with 5–10 CV of 2 M NaCl.
9. Wash the system with 5–10 CV of Milli-Q water.
10. Wash the system with 3–5 CV of 0.1 M HCl.
11. Wash the system with 5–10 CV of Milli-Q water.
12. Wash the system with 3–5 CV of 0.1 M NaOH.
13. Wash system and columns with 5–10 CV of Milli-Q water, followed by 5–10 CV of 20% ethanol.
14. Analyze obtained fractions by SDS-PAGE.

4 Notes

1. Some nanobodies have affinity for protein A or G and therefore protein A or G beads can be used for purification. This enables tag-less protein purification.
2. Take along a mock O/N culture as a negative control.
3. To ensure sufficient aeration, about 2/3 of the flask should be empty. As an example, for production of 800 mL cells, 2 L flask should be used. Terrific broth (TB) can be used alternatively for the production of nanobodies. The difference between YT-2x and TB medium is that the latter is more nutritious and may result in higher yields of produced nanobodies.
4. To evaluate the induction of nanobody, samples can be collected before and after addition of IPTG and analyzed on SDS-PAGE gel. To this end, 1 mL samples should be centrifuged at 4000 × *g* for 5 min and the pellets resuspended in sample buffer. To adjust the ODs, 40 μL of sample buffer should be used per 0.5 of OD. Boil 30 μL of samples at 100 °C for 10 min and load on 15% SDS-PAGE gel. The protein bands are visualized using Coomassie blue staining.
5. Agitation controls dissolved oxygen through automatically controlled agitation speed. When the actual DO_2 value drops below the set point, the system will increase the agitation speed up to as much as the high limit to meet the culture demands. Once the DO_2 set point is reached or exceeded, the agitation will fall back down to the low limit.
6. Fermentor productions can reach very high ODs (up to 20) as a result of better controlled medium for bacterial growth. Due to a longer log phase, IPTG is usually added at an OD between 2 and 4.
7. The beads for affinity chromatography should be chosen based on the tag on the nanobody. Nickel beads are used for purification of nanobodies with histidine-tag (his-tag) and C-tag Affinity Matrix is suitable for the nanobodies with EPEA tag (or C-tag). The right amount of beads required to obtain pure nanobody fractions should be determined experimentally.
8. Depending on the expected nanobody yield, one may choose a 5 mL affinity column with a five times higher binding capacity, instead of a 1 mL column.
9. Air bubbles introduced into a column may clog the buffer flow, resulting in an increased column pressure, and a decreased binding capacity. Therefore, it is important to degas all buffers which are passed over the column. Avoid vigorous shaking of the buffers after degassing.

10. Always check the pH of the periplasm and adjust if necessary (pH should be around 7). Alternatively, the sample may be diluted (e.g., 1:1) in the binding buffer. When purifying a nanobody containing a histidine-tag using a HisTrap column, make sure that the bacterial periplasm does not contain any chelating reagents (e.g., EDTA), as this may remove the nickel ions from the column. This drastically impacts the nanobody yield.
11. The columns can be attached to the system manually, or using a "column attachment program" which is preset for the ÄKTAxpress system. When attaching columns manually, take into account the maximum flow rate and pressure limit for each particular column.
12. When removing ethanol from the system, make sure that sample loops and fractionation tubing are rinsed as well. Additionally, the tubing which will be used for sample loading should be rinsed.
13. Be sure to collect the flow-through during sample loading. When elution fractions are analyzed by SDS-PAGE, the flow-through should be loaded as well. If the flow-through still contains nanobody (if for instance maximum column capacity was reached), an additional purification round can be performed using the flow-through.
14. The maximum recommended sample volume of a 5 mL HiTrap desalting column is 1.5 mL. When two columns are stacked, the maximum sample volume is 3 mL. If the sample volume exceeds 3 mL (high protein yield, or broad elution peak), make sure that any excess sample is collected.
15. When eluted fractions are not clean (multiple bands observed on SDS-PAGE), a shallow linear gradient elution (over 20 CV) may be applied to separate proteins with similar binding strengths. Additionally, mild detergents (e.g., 0.2% Triton X-100) can be added to the elution buffer to reduce possible nonspecific hydrophobic interactions.
16. When purifying the nanobody using a cation-exchange column use the buffer of 0.5–1.5 pH units less than the pI of the nanobody. When purifying the nanobody using an anion-exchange column use the buffer 0.5–1.5 pH units greater than the pI of the nanobody.

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