bio-protocol www.bio-protocol.org/e4406 Bio-protocol 12(09): e4406. DOI:10.21769/BioProtoc.4406

Production and Crystallization of Nanobodies in Complex with the Receptor Binding Domain of the SARS-CoV-2 Spike Protein

Audrey Le Bas[1, #], Halina Mikolajek[2, #], Jiandong Huo[1, 3, #] Chelsea Norman[1], Joshua Dormon[1], James H. Naismith[1, 3, *] and Raymond J. Owens[1, 3, *]

[1]Structural Biology, The Rosalind Franklin Institute, Harwell Science Campus, UK

[2]Diamond Light Source Ltd, Harwell Science Campus, UK

[3]Division of Structural Biology, The Wellcome Centre for Human Genetics, University of Oxford,

[#]Contributed equally to this work

*For correspondence: ray.owens@strubi.ox.ac.uk; james.naismith@strubi.ox.ac.uk

[Abstract] The receptor binding domain (RBD) of the spike protein of SARS-CoV-2 binds angiotensin converting enzyme-2 (ACE-2) on the surface of epithelial cells, leading to fusion, and entry of the virus into the cell. This interaction can be blocked by the binding of llama-derived nanobodies (VHHs) to the RBD, leading to virus neutralisation. Structural analysis of VHH-RBD complexes by X-ray crystallography enables VHH epitopes to be precisely mapped, and the effect of variant mutations to be interpreted and predicted. Key to this is a protocol for the reproducible production and crystallization of the VHH-RBD complexes. Based on our experience, we describe a workflow for expressing and purifying the proteins, and the screening conditions for generating diffraction quality crystals of VHH-RBD complexes. Production and crystallization of protein complexes takes approximately twelve days, from construction of vectors to harvesting and freezing crystals for data collection.

Keywords: SARS CoV-2, Nanobodies, Receptor Binding Domain, Protein purification, Crystallization

[Background] Camelids (llamas, alpacas, and camels) produce a unique type of heavy chain only antibody that comprises variable domains (VHH) linked to Fc constant domains (CH2 and CH3). The VHHs can be produced as single domain antigen-binding proteins or nanobodies (Muyldermans, 2013), with wide applications in the life sciences (Pleiner *et al.*, 2015; Jovcevska and Muyldermans, 2020). In response to the COVID-19 pandemic (Dhama *et al.*, 2020), we and many other groups have developed VHH reagents that bind to the receptor binding domain (RBD) of the SARS-CoV-2 spike protein that block binding to ACE-2 at the cell surface, which is the primary interaction that leads to cell infection (Xiang *et al.*, 2020; Hanke *et al.*, 2020; Schoof *et al.*, 2020; Koenig *et al.*, 2021; Huo *et al.*, 2020, 2021). Multimeric versions of these VHHs, either as trimers or IgG Fc fusions, have been shown to neutralise SARS-CoV-2 both *in vitro* and in animal models of COVID-19 (Huo *et al.*, 2021; Nambulli *et al.*, 2021). Structural analysis of VHH-RBD complexes has revealed two regions where epitopes are clustered, one at or close to the ACE-2 binding surface (cluster 2), and one at the opposite side of the RBD (cluster 1) (Tang *et al.*, 2021). Information about the VHH binding sites has enabled the effect of mutations in the spike protein to be interpreted and predicted. In our experience, forming RBD complexes with two VHH

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

that bind to orthogonal sites has been necessary for the crystallization of some VHH-RBD complexes (Huo *et al.*, 2021).

VHHs are routinely produced in the *E. coli* strain WK6 as hexahistidine tagged proteins, using Isopropyl β-D-1-thiogalactopyranoside (ITPG) inducible promoters, and the addition of a secretion signal (*e.g.*, pelB or ompA), to enable recovery of the VHH from the periplasm following induced expression. Primary purification uses immobilised metal affinity chromatography (IMAC) with gel filtration added as a polishing step. Alternatively, VHHs can be directly purified from lysed cells though, in our experience, periplasmic extraction by osmotic shock gives higher protein yields.

As a glycosylated protein, the production of the RBD of the SARS-CoV-2 spike protein requires expression in higher eukaryotic cells for which both insect and mammalian cells have been used. An appropriate signal sequence is added to the N-terminus to direct product to the cell media, and a hexahistidine tag added to the C-terminus for purification by IMAC. The N-glycosylation of the RBD (Asn331 and Asn343) introduces chemical heterogeneity, particularly if produced in mammalian cells, which generally inhibits crystallization (Chang *et al.*, 2007). By growing cells in the presence of the mannosidase inhibitor kifunensine, N-glycosylation can be arrested at a high mannose state (GlcNAc2Man9 glycoforms). These can subsequently be trimmed back to single N-acetylglucosamine residues, by treatment with endoglycosidase F1 or H (Chang *et al.*, 2007). Based on past experience (Nettleship *et al.*, 2013), this is most efficiently carried out prior to purification of the VHH-RBD complex.

An issue for the purification of secreted glycoproteins from cell culture media by IMAC is that some media components displace the His-tagged protein-of-interest during the chromatography step, significantly reducing the yield. Here, we have used an automated method of affinity purification by IMAC and gel filtration, which involves loading the sample onto the Ni-NTA column in batches, with a column washing step between each batch (Nettleship *et al.*, 2009).

We have reported the crystallization of a number of VHH-RBD complexes using commercially available screens, in 96-well format, and low sample volumes of 1–200 nL. Diffraction data were collected from the primary crystal hits using synchrotron X-rays, and structures solved by molecular replacement (Huo *et al.*, 2020, 2021). Key to achieving good quality crystals was the preparation of the proteins. Thus, in this article, we describe our optimised workflow for producing VHH-RBD complexes and their crystallization for analysis by X-ray crystallography.

Materials and Reagents

A. Production of a receptor binding domain

1. Vector construction
   a. pOPINTTG vector digested with KpnI/PmeI (Figure 1A)
   b. Human codon optimised synthetic RBD gene (encoding amino acids 330–352) with 15-bp extensions overlapping with pOPINTTG in-fusion entry sites (lower case):
   5’gcgtagctgaaaccggcCCGAATATCACAAATCTTTGTCCTTTCGGAGAAGTATTCAACGCA
   ACTCGCTTCGCATCAGTATATGCCTGGAACCGCAAACGAATTTCTAATTGTGTCGCCG

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

ACTACTCTGTGCTTTACAACTCAGCATCATTTTCAACATTCAAATGCTATGGTGTCTCCC
CTACGAAGTTGAACGATCTTTGTTTCACTAATGTCTACGCCGATTCCTTTGTTATTAGG
GGGGACGAGGTTCGCCAGATCGCGCCGGGCCAAACGGGCAAGATAGCTGACTATAA
TTATAAGCTGCCAGACGACTTTACAGGCTGCGTTATCGCTTGGAATTCAAACAATCTTG
ATAGCAAAGTGGGTGGTAATTATAACTACCTCTACAGACTGTTTCGCAAGTCTAATCTG
AAGCCTTTCGAGCGGGACATCTCTACTGAGATCTATCAAGCTGGTTCAACCCCCTGCA
ATGGCGTCGAGGGTTTTAACTGTTACTTCCCACTTCAGTCATACGGATTTCAACCAACT
AATGGGGTTGGCTATCAGCCGTACCGCGTGGTCGTTCTTAGCTTTGAGCTGCTTCAC
GCCCCTGCAACGGTGTGCGGACCGAAAAAAAGTACAAAaaacatcaccatcac 3’

c. ClonExpress II One Step Cloning kit (Vazyme, catalog number: C113-02)
d. NucleoSpin 148® Gel and PCR Clean-up kit (MACHEREY-NAGEL, catalog number: 12303368)
e. Stellar competent cells (Takara Bio, catalog number: 636766)
f. LB medium (Sigma-Aldrich, catalog number: L3022)
g. S.O.C. (Super Optimal broth with Catabolite repression) recovery medium (ThermoFisher Scientific, catalog number: 15544034)
h. Ampicillin (Sigma-Aldrich, catalog number: A9393)
i. LB-agar plates supplemented with 100 µg/mL ampicillin
j. Plasmid miniprep kit (*e.g.*, Qiagen, catalog number: 27104)
k. Plasmid Plus Midi kit (*e.g.*, Qiagen, catalog number: 12941)
l. Sequencing primers: pTTfwd 5’ TCCACAGGTGTCCACTCC 3’
pTTrev 5’ TCCTTTATTAGCCAGAGG 3’

2. Expression in expi293™ cell
   a. 500 mL sterile baffled flasks with vented closure (ThermoFisher Scientific, catalog number: 4116-0500)
   b. Countess™ cell counting chamber slides (ThermoFisher Scientific, catalog number: C10228)
   c. Expi293™ cells (ThermoFisher Scientific, catalog number: A14527)
   d. Expi293™ expression medium (ThermoFisher Scientific, catalog number: A1435101)
   e. Trypan blue solution (ThermoFisher Scientific, catalog number: 15250061)
   f. Hanks' Balanced Salt Solution (HBSS) (10×) (ThermoFisher Scientific, catalog number: 14185052)
   g. Gibco OPTI-MEM reduced serum medium (ThermoFisher Scientific, catalog number: 31985062)
   h. Valproic acid (Sigma-Aldrich, catalog number: P4543)
   i. Glucose (45% solution) (see Recipes or from Sigma-Aldrich, catalog number: G8769)
   j. Sodium propionate (Sigma-Aldrich, catalog number: P1880)
   k. PEI MAX 40 K (Polysciences Inc., catalog number: 24,765-1)
   l. Kifunensine (Sigma-Aldrich, catalog number: K1140)

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

m. Pre-cast SDS polyacrylamide gels (*e.g.*, NuPAGE™ 10%, Bis-Tris ThermoFisher Scientific, catalog number WG1201A)
n. InstantBlue® Coomassie protein stain (Abcam, catalog number: ab119211)

3. RBD purification
   a. 5 ml HisTrap_FF Ni-NTA columns (Cytiva, catalog number: 1752860)
   b. SD75 16/600 size exclusion column (Cytiva, catalog number: 28989333)
   c. Imidazole (Sigma-Aldrich, catalog number: I2399)
   d. PBS, Phosphate Buffered Saline, 10× Solution (Fisher, catalog number: BP399-20)
   e. Wash buffer (see Recipes)
   f. Elution buffer (see Recipes)
   g. Gel filtration buffers (see Recipes)

B. VHH production

1. Vector construction
   a. pADL-23c vector cut with SfiI (Figure 1B) (New England Biolabs, catalog number: R0123S)
   b. Infusion primers:
      VHH Fwd primer 5' GTTATTACTCGCGGCCCAGCCGGCCATGGCCC 3'
      VHH Rev primer 5' GGTGATGGTGTTGGCCTTTATTAATGATGGTGGTGATGGTG 3'
   c. Proof reading polymerase (*e.g.*, PhusionFlash™ high fidelity polymerase ThermoFisher Scientific, catalog number: F548S)
   d. pADL-23c sequencing primers: PhDseqFwd 5' GCTTCCGGCTCGTATGTTG 3'
      PhDseqRev 5' GTCGTCTTTCCAGACGTTAG 3'
   e. 2 mL Cryovials (*e.g.*, ThermoFisher Scientific, catalog number: 5000-0020)
2. *E. coli* expression
   a. WK6 cells *Escherichia coli* (Migula) Castellani and Chalmers (ATCC, catalog number: 47078)
   b. Terrific Broth (TB) Medium (see Recipes)
   c. Isopropyl β-D-1-thiogalactopyranoside, IPTG (Sigma-Aldrich, catalog number: I6758)
   d. Glucose
   e. Magnesium chloride
   f. Ampicillin
3. VHH purification
   a. TES buffer (see Recipes)
   b. DNase I (Sigma-Aldrich, catalog number: D4263)
   c. Magnesium Chloride (Sigma-Aldrich, catalog number: M8266)
   d. Sucrose (Sigma-Aldrich, catalog number: S0389)

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

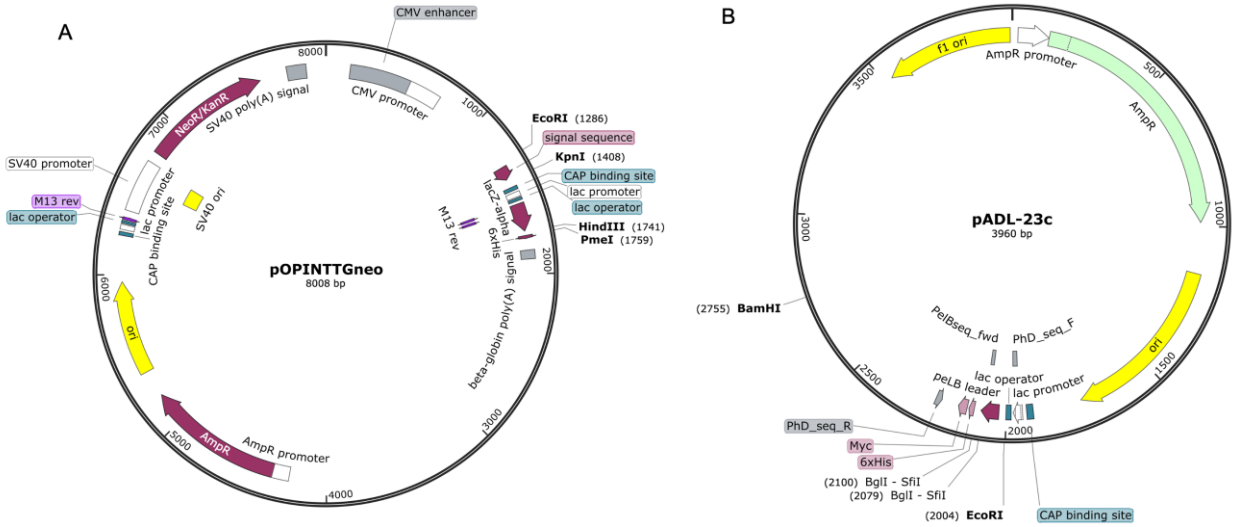

Figure 1. Vector maps (A) pOPINTTGneo (B) pADL-23c

C. Crystallization of VHH-RBD complexes

1. Preparation of VHH-RBD complexes
   a. EndoH (New England Biolabs, catalog number: P0702S)
   b. SD200 10/300 size exclusion column (Cytiva, catalog number: 28990944)
2. Crystallization screening
   a. Swisssci Triple-Drop crystallization plates (Molecular Dimensions, catalog number: MD11-003-100)
   b. V well Microplate without lid, natural, polypropylene (Greiner, catalog number: 651201 96)
   c. VIEWseal™ plate sealer, transparent, non-piercable Greiner, catalog number: 676070)
   d. Pact premier™ crystallization screen condition (Molecular Dimension, catalog number: MDSR-29)
   e. JCSG-plus™ crystallization screen condition (Molecular Dimensions, catalog number: MDSR-37)
   f. SG1™ crystallization condition (Molecular Dimensions, catalog number: MDSR-88)
3. Cryopreservation of crystals
   a. Glycerol (Molecular Dimensions, catalog number: MD2-100-65)
   b. PEG 400 (Sigma-Aldrich, catalog number: 06855)
   c. Mounted Round LithoLoops (0.25 mm) (Molecular Dimensions, catalog number: MD7-137)
   d. Mounted Round LithoLoops (0.15 mm) (Molecular Dimensions catalog number: MD7-135)
   e. Standard Foam Dewar (Molecular Dimensions, catalog number: MD7-35)
   f. Uni-Puck 10 Pack (Molecular Dimensions, catalog number: MD7-613)
   g. Cryotool set (Molecular Dimensions, catalog number: MD7-517)
   h. Dry Shipper (Molecular Dimensions, catalog number: MD7-21

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

Equipment

1. Microvolume spectrophotometer (*e.g.*, ThermoFisher Scientific, NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer: ND-ONE-W)
2. CO_2 orbital shaker (*e.g.*, n-Biotek.com ANICELL incubator, catalog number: NB-206CXL/NB)
3. Tabletop centrifuge suitable for 50 mL Falcon tubes (Sorvall Legend RT Plus)
4. Cell Counter (*e.g.*, ThermoFisher Scientific Countess™ 3 Automated Cell Counter)
5. ÄKTA Xpress automated multi-step purification system
6. Liquid handler (*e.g.*, Hydra Dispenser by Art Robbins Instruments)
7. Low volume dispenser for crystallization plates (*e.g.*, SPT labtech mosquito® LV)
8. Crystal plate imager (*e.g.*, Formulatrix RockImager® system)

Procedure

A. Production of receptor binding domains

1. Vector construction
    a. For 10 µL of in-fusion reaction, mix 20 ng (1–3 µL) of synthetic gene, 100 ng (1–2 µL) of PmeI/NcoI cut pOPINTTG vector, 1 µL of Exnase II, and 2 µL of optimized buffer supplied with the cloning kit.
    b. Incubate the reaction at 37°C for 30 min, and then stop immediately, by adding 20 µL of ice-cold TE buffer.
    c. Use 5 µL of the resulting reaction mixture to transform 20 µL of Stellar competent cells, by incubating on ice for 30 min, then heat shocking at 42°C for 45 s.
    d. Add 400 µL of SOC media and incubate at 37°C for 45 min.
    e. Plate 100 µL of the cell and media mixture on LB-agar plates supplemented with 100 µg/mL ampicillin, and culture at 37°C overnight.
    f. Pick single colonies into 3 mL of LB containing 100 µg/mL ampicillin and incubate at 37°C overnight.
    g. Produce glycerol stocks, by adding 0.5 mL of 50% (v/v) sterile glycerol to 0.5 mL of overnight culture of cells in LB in a 2-mL cryovial, and freezing at -80°C.
    h. Prepare DNA from pelleted cells, and confirm correct clones by sequencing minipreps DNAs with pTTfwd and PTTrev primers.
    i. Use 50% glycerol stock to grow larger scale cultures for transfection-grade plasmid preparation.
    j. Purify transfection-grade plasmids (0.5–1 mg) from 150 mL of overnight LB culture, using QIAGEN Plasmid Plus Midi kit and, if required, store plasmids at -20°C in sterile 1.5-mL Eppendorf tubes.
    k. Measure the purity and concentration of DNA using NanoDrop spectrophotometer. Plasmid DNA used for transfections should be of high purity (see Note 1).

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

l. The concentration and purity of DNA is calculated automatically by the instrument using the following equations:
Concentration: DNA (μg/mL) = (A_{260} reading – A_{320} reading) × dilution factor × 50 μg/mL
Purity: (A_{260}/A_{280}) = (A_{260} reading – A_{320} reading) ÷ (A_{280} reading – A_{320} reading)

m. Use 1 μg DNA per one million of transfected cells.

2. Transfection protocol

a. Maintain the suspension culture of Expi293™ cells for at least three passages after defrosting (passage numbers 3–30 can be used in experiments) in a humidified (80%) incubator, with 5–8% CO_2, at 37°C, on an orbital shaker at 120 rpm in Gibco Expi293™ Express medium, at a cell density between 0.5 and 5.0 × 10^6 cells/mL. Use a 125-mL flask to maintain 30 mL of Expi293 cells.

b. One day before transfection, seed Expi293™ cells at a cell density of 1 × 10^6 cells/mL. For each preparation of RBD, set up three cultures of 170 mL in 500-mL flasks.

c. On the day of transfection, determine cell count and viability, and if cell count is between 2 × 10^6–2.5 × 10^6/mL and at least 95% viable, proceed with transfection (see Note 2).

d. For each 170-mL culture, mix 17 mL of OPTI-MEM media with 170 μg plasmid DNA and 918 μL of PEI Max 40kDa transfection reagent in a 50-mL Falcon tube.

e. Mix thoroughly, incubate at room temperature (RT) for 10 min, and add gently (dropwise) to the Expi293™ cells (see Note 3).

f. Add kifunensine from a stock concentration of 1 mg/mL (100 μL per 100 mL of culture volume), and return cells to incubate on the orbital shaker at 125 rpm, 5–8% CO_2, 80% humidity and 37°C.

g. After 16–18 h, add to each 170 mL culture, 2,890 μL of valproic acid, 1,100 μL of sodium propionate, and 3,400 μL of glucose. Return the culture to the incubator (see Note 4).

h. On day 5 after transfection, determine cell count and viability.

i. Harvest media, and spin in a 0.5-L centrifuge bottle at 6,000 × *g* for 20 min. Filter sterilise using a 0.45 μm 0.5-L bottle top filter.

3. Purification

The IMAC-SEC purification protocol is for an ÄKTA Xpress platform using a programme described in Nettleship *et al*. (2009). The same workflow can be implemented on other purification systems with automated peak detection. A transcript of the Unicorn programme is provided as an Appendix.

a. Equilibrate the gel filtration column using the “Gel Filtration Equilibration” programme and the Gel filtration buffer (20 mM Tris, pH 7.5, 200 mM NaCl).

b. Follow the method “Mammalian Prepping System” to set up the system. It will pump wash A1 and A2, and clean the inlets. It will then ask you to screw the 5-mL column into position 1, and will equilibrate the column.

c. Add an equal volume of PBS to the filtered cell supernatant, and adjust the pH to 7.4 with NaOH.

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

d. Once the column is plugged in and the sample is ready to be loaded, slowly remove the A2 line from the buffer (by small movements to avoid bubbles in the line), and insert it into the sample. Make sure all the lines are at the bottom of the bottles.
e. Leave the protocol to run overnight.
f. Using the trace from the ÄKTA, select the correct fractions containing the protein of interest. Mix 10 µL of protein solution with 10 µL of sample buffer, and heat at 95°C for 5 min. Run an SDS-PAGE gel of the samples, and stain it using Instant Blue®.
g. Concentrate the protein, usually to 5 mg/mL, using a 10 kDa concentrator centrifuge at 2,500 × *g* and 4°C.
h. Aliquot (*e.g.*, 0.1 mL) and flash freeze the protein, by plunging tubes into liquid nitrogen.
i. Store at -80°C.

B. Production of VHHs

1. Vector construction

   VHHs identified by screening M13 phage display libraries are re-expressed for protein production.

   a. Amplify VHH from source display vector using VHH Fwd primer and VHH Rev primers and the following PCR conditions:
      1) 98°C for 10 s
      2) 30 cycles of:
         98°C for 1 s
         60°C for 5 s
         72°C for 15 s
      3) 72°C for 2 min
      4) 4°C hold
   b. Purify amplified VHH DNA by agarose gel electrophoresis, and extract using the Nucleospin® kit according to the manufacturer's instructions. Clone PCR product into the SfiI cut pADL-23c vector, as described for the cloning of the RBD.
   c. Verify clones, by sequencing with PhDseqFwd and PhDseqRev primers.

2. Expression in *E. coli*

   a. Transform chemically competent WK6 cells as described above, for construction of the RBD expression vector.
   b. Pick a colony from the plate of transformed cells, and set up a pre-culture: 8 mL of TB supplemented with 100 µg/mL ampicillin, 2% glucose and 1 mM $MgCl_2$.
   c. Set up the culture: 800 mL of TB medium in a 2-L flask supplemented with 100 µg/mL ampicillin, 0.1% glucose, and 1 mM $MgCl_2$.
   d. Pre-warm the flask/medium to 37°C.
   e. Add pre-culture and grow culture on an orbital shaker at 225 rpm and 37°C.

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

f. Add IPTG to a final concentration of 1 mM when OD_{600} reaches ~1.2 (usually takes around 3.5 h), and continue growing at 225 rpm and 28°C overnight.
g. Pellet the cells at 2,500 × *g* at 4°C for 15 min.

3. Purification of VHHs
   a. Add 15 mL of TES buffer to the cell pellet, and resuspend slowly in a bottle with a magnetic stirrer at 4°C overnight.
   b. The next day, add twice the volume of *cold* TES/4 buffer supplemented with 120U Kunitz DNaseI to the resuspended culture, and slowly stir for 2 h.
   c. Top up to 80-mL with TES/4 buffer.
   d. Spin down the pellet at 28,000 × *g* and 4°C for 30 min (in a 50-mL tube).
   e. Filter the supernatant through a 0.8 µm filter under vacuum.
   f. Dilute the supernatant with five volumes of PBS (pH 7.4), and mix well.
   g. Load the sample onto two 5-mL IMAC columns connected in series, at <2 mL/min.
   h. Wash the column with PBS (pH 7.4) containing 30 mM imidazole.
   i. Elute the sample with PBS containing 300 mM imidazole at 1 mL/min into a 96-well collection plate, collecting 1-mL fractions.
   j. Pool A_{280} peak fractions (approximately 7.5 mL) and run on a Superdex S75 16/600 in gel filtration buffer (50 mM Tris pH 7, 150 mM NaCl).
   j. Pool A_{280} peak protein fractions, and concentrate using a 5 kDa MWCO concentrator, usually to 15 mg/mL (see Note 5).
   k. Aliquot (*e.g.*, 0.1 mL) and flash freeze the protein, by plunging tubes into liquid nitrogen.
   l. Store at -80°C.

C. Crystallization of VHH-RBD complexes

1. Preparation of VHH:RBD complexes
   a. Mix 5 mg of RBD (5 mg/mL) with 3 mg VHH (15 mg/mL) at a molar ratio RBD:VHH of 1:1.2, and incubate under agitation at 2 rpm in a cold room for 3 h (see Note 6).
   b. Incubate the RBD-VHH complex with 0.4 mg of EndoH glycosidase (1 mg/mL) under agitation at 2 rpm at room temperature overnight (see Note 7).
   c. Concentrate the mixture to 1 mL with a 5 kDa MWCO concentrator, and inject onto a Superdex 200 10/300 in gel filtration buffer (50 mM Tris pH 7, 150 mM NaCl).
   d. Monitor A_{280}, pool peak fractions, and concentrate using 5 kDa MWCO concentrator to 20 mg/mL (see Figures 2 and 3).

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

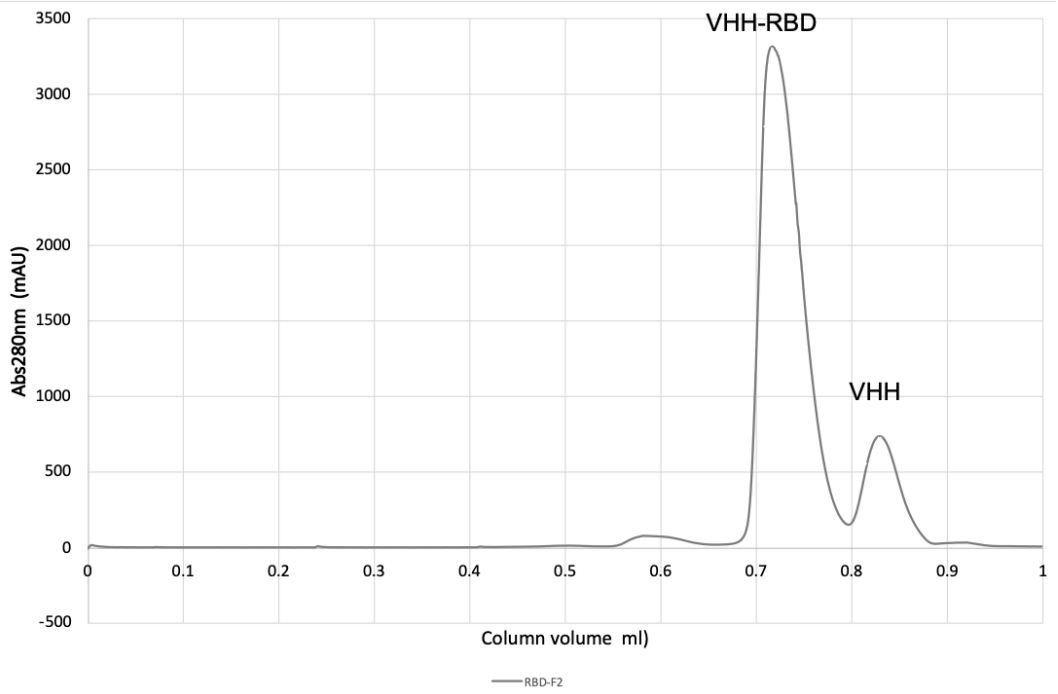

Figure 2. Purification of VHH-RBD complex on a SD200 20/300 size exclusion column (CV 23.56 mL).

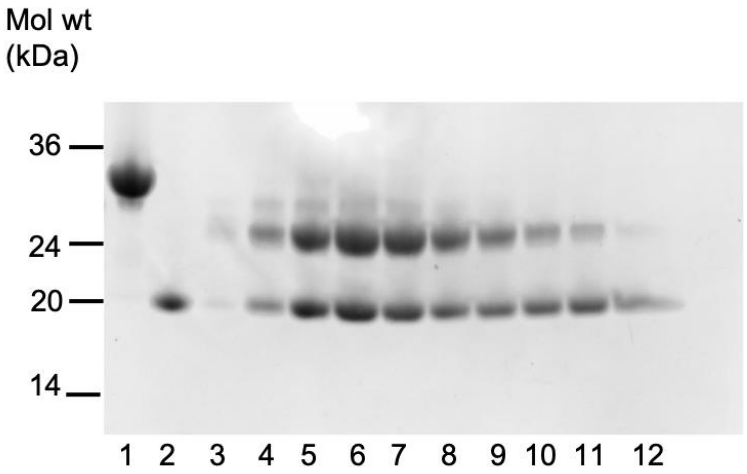

Figure 3. SDS-Page of RBD (lane 1), VHH (lane 2), and fractions from gel filtration of RBD-VHH complexes, following treatment with endoglycosidase HH (lanes 3–12).

2. Crystallization screening
   a. Dispense 25 µL of crystallization solution from 96-well masterblock into the reservoir well of Swisssci crystal plate, using the Hydra liquid handler.
   b. Pipette the protein solution into a column in a V well microplate well plate.

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

c. Set up a sitting drop plate comprising 288 drops, using the robotic liquid handler (Mosquito) using three protein:reservoir ratios (100 nL protein + 100 nL reservoir; 200 nL protein + 100 nL reservoir; 100 nL protein + 200 nL reservoir) (see Note 8).
d. Seal completed plate using VIEWseal.
e. Image and store plates by Fibonacci schedule in Formulatrix imager at 20°C (see Figure 4 for examples of crystals).
f. Cut out a square of seal over the selected crystal drop, and open drop.
g. Add 1 µL of cryoprotectant mix (30% glycerol or Peg 400 in original crystallization condition) onto crystals/drop. Table 1 gives examples of crystallized VHH-RBD complexes.
h. Place mounting pin onto magnetic wand, loop crystal from drop, and place rapidly into foam dewar containing liquid nitrogen.
i. Store mounted crystal in puck within foam dewar.
j. Transfer puck into liquid nitrogen pre-cooled Dry shipper.

Table 1. Examples of crystallized VHH-RBD complexes with details of crystallization conditions, the space group, and resolution limit of crystals generated.

Complex	**RBD-F2**	**RBD-H3-C1**	**RBD-C5**
Crystal screen	Pact™	JCSG™	SG1™
Crystallisation condition	0.1 M SPG, pH 8, 25% Peg 1500	1.0 M Lithium chloride, 0.1 M Citrate pH 4, 20% Peg 6000	0.2 M Na Acetate, 0.1 M Na Cacodylate pH 6.5 and 30% w/v PEG 8000
Time for crystal formation	3 days	less than 24 h	3 days optimal growth
Ratio	0.2 µL protein with 0.1 µL reservoir	0.2 µL protein with 0.1 µL reservoir	0.1 µL protein with 0.1 µL reservoir
Concentration	34 mg/mL	18 mg/mL	18 mg/mL
Cryoprotectant	glycerol	Peg400	glycerol
Data collection			
Exposure	0.006s	0.012s	0.008s
Space group	P31	P41212	P21212
Resolution (Å)	2.3	1.9	1.5

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

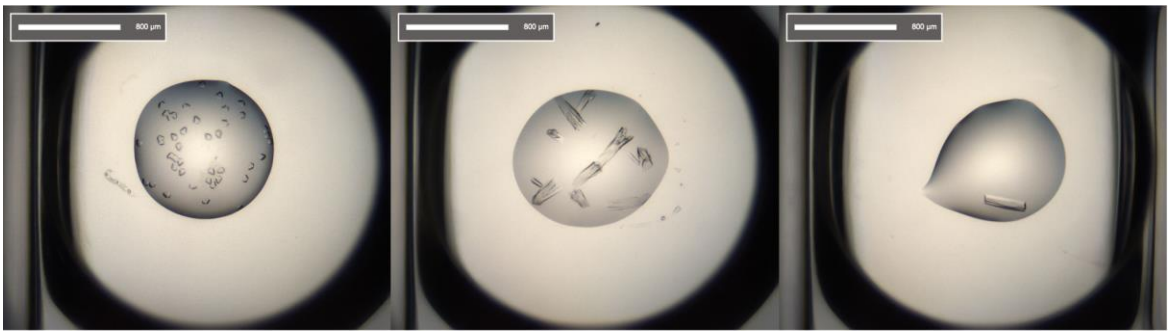

Figure 4. Crystal shape of the different complexes: RBD-F2 (left), RBD-H3-C1 (middle), and RBD-C5 (right).

Notes

1. Good quality DNA with minimum protein and chemical contamination should have ratios of absorbance 260/280 between 1.8–2.0, and 260/230 between 2.0–2.2.
2. Our protocol is suitable for any scale of expression: 1–3 mL plate experiments, and 30–300 mL up-scaled expression in flasks. Scale provided volumes and quantities of reagents proportionally to the volume of transfected cells.
3. Do not mix DNA and PEI directly, as they will precipitate immediately.
4. The use of valproic acid, sodium propionate (histone deactylase inhibitors), and glucose feed in combination helps substantially enhance gene expression.
5. It is important to check periodically that there is no precipitation of the VHH during the concentration step and to stop further concentrating the protein if this is observed. Any precipitate can be removed by centrifugation of the sample in a microfuge at 12,000 × *g* for 10 min and then measuring the final protein concentration by absorbance at 280 nm.
6. A molar excess of VHH is added, to ensure that the RBD is fully complexed with the nanobody.
7. EndoH has maximum efficiency at pH ~5.2. However, as the majority of proteins are unstable at this pH, the reaction is carried out at pH 7.5. Also, many proteins are unstable at 37°C for long periods of time, so RT is preferred for the reaction. EndoH is active at RT and at pH 7.5, but the reaction takes longer than it would if it was run under optimal conditions.
8. The two sparse matrix screens (JCSG+ and SG1) were routinely screened at two different concentrations (usually, 34 mg/mL, and 18 mg/mL) with three different ratios. For the complexes RBD-F2 and RBD-H3-C1, we obtained a number of crystallization hits, while for RBD-C5 we only obtained one hit. Only occasionally the PACT screen was used. Crystals were harvested straight from the sparse matrix screen and did not require further optimization. To obtain more crystals with very subtle different diffraction quality, usually the same condition was set up as a row on the mosquito, using the same protocol. This also allowed testing of two different cryo-conditions (usually, glycerol, and Peg 400). Crystals were very reproducible. The cleanest crystals were harvested from all hits usually within 24 h of growth, but diffraction quality remained stable up to 5 days thereafter for most hits.

Bio-protocol 12(09): e4406.
DOI:10.21769/BioProtoc.4406

Recipes

1. Valproic acid
   500 mg in 10 mL of cell media or HBSS, filter 0.2 µm, and store at -20°C.
2. Propionate solution
   1 g sodium propionate in 10 mL of cell media or HBSS, filter, and store at -20°C.
3. PEI
   Suspend 100 mg of PEI Max 40K in 90 mL of MilliQ water. Stir using a PTFE coated stirring bar. It should take less than 5 min to dissolve, then adjust the pH using NaOH or HCl to 7.0. Add water to 100 mL, filter 0.2 µm, and store at -20°C.
4. Glucose
   45 g in 100 mL of HBSS or cell medium, filter 0.2 µm, and store at -20°C.
5. Wash buffer
   PBS + 30 mM imidazole pH 7.4
6. Elution buffer
   PBS + 300 mM imidazole pH 7.4
7. Gel filtration buffers
   20 mM Tris, and pH 7.5, 200 mM NaCl, or 50 mM Tris pH 7, 150 mM NaCl
8. TES buffer
   200 mM Tris pH 8, 0.5 mM EDTA, 500 mM sucrose
9. TES/4 buffer
   50 M Tris pH 8, 125 mM sucrose
10. TB medium
    Yeast extract (24 g/L), Tryptone (20 g/L), Glycerol (4 mL/L), Phosphate buffer (100 mL/L) of 0.17 M KH_2PO_4, 0.72 M K_2HPO_4

Acknowledgments

This work was supported by the Rosalind Franklin Institute, funding delivery partner EPSRC. and the Rosalind Franklin Institute EPSRC grant no. EP/ S025243/1. J.H.N., A.L.B. are supported by Wellcome Trust (100209/Z/12/Z). J.H. is supported by the EPA Cephalosporin and Edward Penley Abraham Funds. X-ray data were obtained using Diamond Light Source COVID-19 Rapid Access time on Beamline I03, I04 and I24 (proposal MX27031).

Competing interests

The Rosalind Franklin Institute has filed a patent on the identification of nanobodies to the spike protein of SARS-CoV-2; R.J.O., J.H. and J.H.N. are named as inventors. The other authors declare no competing interest.

References

1. Chang, V. T., Crispin, M., Aricescu, A. R., Harvey, D. J., Nettleship, J. E., Fennelly, J. A., Yu, C., Boles, K. S., Evans, E. J., Stuart, D. I., *et al*. (2007). Glycoprotein structural genomics: solving the glycosylation problem. *Structure* 15(3): 267-273.
2. Dhama, K., Khan, S., Tiwari, R., Sircar, S., Bhat, S., Malik, Y. S., Singh, K. P., Chaicumpa, W., Bonilla-Aldana, D. K. and Rodriguez-Morales, A. J. (2020). Coronavirus Disease 2019-COVID-19. *Clin Microbiol Rev* 33(4): e00028-20.
3. Hanke, L., Vidakovics Perez, L., Sheward, D. J., Das, H., Schulte, T., Moliner-Morro, A., Corcoran, M., Achour, A., Karlsson Hedestam, G. B., Hallberg, B. M., *et al*. (2020). An alpaca nanobody neutralizes SARS-CoV-2 by blocking receptor interaction. *Nat Commun* 11(1): 4420.
4. Huo, J., Le Bas, A., Ruza, R. R., Duyvesteyn, H. M. E., Mikolajek, H., Malinauskas, T., Tan, T. K., Rijal, P., Dumoux, M., Ward, P. N., *et al*. (2020). Neutralizing nanobodies bind SARS-CoV-2 spike RBD and block interaction with ACE2. *Nat Struct Mol Biol* 27(9): 846-854.
5. Huo, J., Mikolajek, H., Le Bas, A., Clark, J. J., Sharma, P., Kipar, A., Dormon, J., Norman, C., Weckener, M., Clare, D. K., *et al*. (2021). A potent SARS-CoV-2 neutralising nanobody shows therapeutic efficacy in the Syrian golden hamster model of COVID-19. *Nat Commun* 12(1): 5469.
6. Jovcevska, I. and Muyldermans, S. (2020). The Therapeutic Potential of Nanobodies. *BioDrugs* 34(1): 11-26.
7. Koenig, P. A., Das, H., Liu, H., Kummerer, B. M., Gohr, F. N., Jenster, L. M., Schiffelers, L. D. J., Tesfamariam, Y. M., Uchima, M., Wuerth, J. D., *et al*. (2021). Structure-guided multivalent nanobodies block SARS-CoV-2 infection and suppress mutational escape. *Science* 371(6530): eabe6230.
8. Muyldermans, S. (2013). Nanobodies: natural single-domain antibodies. *Annu Rev Biochem* 82: 775-797.
9. Nambulli, S., Xiang, Y., Tilston-Lunel, N. L., Rennick, L. J., Sang, Z., Klimstra, W. B., Reed, D. S., Crossland, N. A., Shi, Y. and Duprex, W. P. (2021). Inhalable Nanobody (PiN-21) prevents and treats SARS-CoV-2 infections in Syrian hamsters at ultra-low doses. *Sci Adv* 7(22): eabh0319.
10. Nettleship, J. E., Rahman-Huq, N. and Owens, R. J. (2009). The production of glycoproteins by transient expression in Mammalian cells. *Methods Mol Biol* 498: 245-263.
11. Nettleship, J. E., Ren, J., Scott, D. J., Rahman, N., Hatherley, D., Zhao, Y., Stuart, D. I., Barclay, A. N. and Owens, R. J. (2013). Crystal structure of signal regulatory protein gamma (SIRPgamma) in complex with an antibody Fab fragment. *BMC Struct Biol* 13: 13.

12. Pleiner, T., Bates, M., Trakhanov, S., Lee, C. T., Schliep, J. E., Chug, H., Bohning, M., Stark, H., Urlaub, H. and Gorlich, D. (2015). Nanobodies: site-specific labeling for super-resolution imaging, rapid epitope-mapping and native protein complex isolation. *Elife* 4: e11349.
13. Schoof, M., Faust, B., Saunders, R. A., Sangwan, S., Rezelj, V., Hoppe, N., Boone, M., Billesbolle, C. B., Puchades, C., Azumaya, C. M., *et al*. (2020). An ultrapotent synthetic nanobody neutralizes SARS-CoV-2 by stabilizing inactive Spike. *Science* 370(6523): 1473-1479.
14. Tang, Q., Owens, R. J. and Naismith, J. H. (2021). Structural Biology of Nanobodies against the Spike Protein of SARS-CoV-2. *Viruses* 13(11): 2214.
15. Wec, A. Z., Wrapp, D., Herbert, A. S., Maurer, D. P., Haslwanter, D., Sakharkar, M., Jangra, R. K., Dieterle, M. E., Lilov, A., Huang, D., *et al*. (2020). Broad neutralization of SARS-related viruses by human monoclonal antibodies. *Science* 369(6504): 731-736.
16. Xiang, Y., Nambulli, S., Xiao, Z., Liu, H., Sang, Z., Duprex, W. P., Schneidman-Duhovny, D., Zhang, C. and Shi, Y. (2020). Versatile and multivalent nanobodies efficiently neutralize SARS-CoV-2. *Science* 370(6523): 1479-1484.

