A general protocol for the generation of Nanobodies for structural biology

Els Pardon\textsuperscript{1,2}, Toon Laeremans\textsuperscript{1,2}, Sarah Triest\textsuperscript{1,2}, Søren G F Rasmussen\textsuperscript{3}, Alexandre Wohlkönig\textsuperscript{1,2}, Armin Ruf\textsuperscript{4}, Serge Muyldermans\textsuperscript{2,5}, Wim G J Hol\textsuperscript{6}, Brian K Kobilka\textsuperscript{7} \& Jan Steyaert\textsuperscript{1,2}

\textsuperscript{1}Structural Biology Brussels, Vrije Universiteit Brussel (VUB), Brussels, Belgium. \textsuperscript{2}Structural Biology Research Center, Vlaams Instituut voor Biotechnologie (VIB), Brussels, Belgium. \textsuperscript{3}Department of Neuroscience and Pharmacology, The Panum Institute, University of Copenhagen, Copenhagen, Denmark. \textsuperscript{4}Pharma Research and Early Development (pRED), Small Molecule Research, Discovery Technologies, F. Hoffmann-La Roche, Basel, Switzerland. \textsuperscript{5}Cellular and Molecular Immunology, VUB, Brussels, Belgium. \textsuperscript{6}Department of Biochemistry, Biomolecular Structure Center, School of Medicine, University of Washington, Seattle, Washington, USA. \textsuperscript{7}Department of Molecular and Cellular Physiology, School of Medicine, Stanford University, Stanford, California, USA. Correspondence should be addressed to J.S. (jan.steyaert@vib-vub.be).

Published online 27 February 2014; doi:10.1038/nprot.2014.039

There is growing interest in using antibodies as auxiliary tools to crystallize proteins. Here we describe a general protocol for the generation of Nanobodies to be used as crystallization chaperones for the structural investigation of diverse conformational states of flexible (membrane) proteins and complexes thereof. Our technology has a competitive advantage over other recombinant crystallization chaperones in that we fully exploit the natural humoral response against native antigens.

Accordingly, we provide detailed protocols for the immunization with native proteins and for the selection by phage display of in vivo–matured Nanobodies that bind conformational epitopes of functional proteins. Three representative examples illustrate that the outlined procedures are robust, making it possible to solve by Nanobody-assisted X-ray crystallography in a time span of 6–12 months.

INTRODUCTION

The production of diffraction-quality crystals remains the major bottleneck in macromolecular X-ray crystallography. Collective efforts of several laboratories have demonstrated that Nanobodies are exquisite chaperones for crystallizing complex biological systems such as membrane proteins\textsuperscript{1–3}, transient multiprotein assemblies\textsuperscript{2,4–6}, transient conformational states\textsuperscript{4} and intrinsically disordered proteins\textsuperscript{7,8}. Further, they can be used as structural probes of protein misfolding and fibril formation\textsuperscript{9,10}. Nanobodies are the small (15 kDa) and stable single-domain fragments harboring the full antigen-binding capacity of the original heavy chain–only antibodies that naturally occur in camels against native antigens. To our knowledge, there are no other recombinant crystallization chaperones that bind conformational epitopes with high affinity, they perform poorly at binding disordered proteins, such as Fabs or scFvs. Because of their compact shape, Nanobodies expose a convex paratope and have access to cavities or clefts on the surface of proteins\textsuperscript{11,12} that are often inaccessible to conventional antibodies. These cryptic epitopes can be readily recognized by the long CDR3 loop of the Nanobody.

In our experience, Nanobodies raised in vivo by immunization against, and selected on, properly folded proteins systematically recognize discontinuous amino acid segments of the native protein conformation (i.e., conformational epitopes), making them ideal tools to selectively stabilize specific conformational states of (membrane) proteins.

For the discovery of Nanobodies as crystallization chaperones, \textsim\ 1 mg of functional protein is required. The generation of in vivo–matured Nanobodies can therefore be incorporated in the crystallization pipeline even before the purification of the protein has been fully optimized and scaled up. Nanobodies binding conformational epitopes (conformational Nanobodies) can subsequently be used for preparing pure, homogeneous and highly concentrated monodisperse samples that are required for crystallization\textsuperscript{15}. If no native purified protein is available, genetic and cell-based vaccinations, combined with cell-based selection approaches, have been successfully applied in our laboratory and elsewhere to generate Nanobodies against target proteins in their native conformation\textsuperscript{16–18}.

Comparisons with other approaches

Here we present a general protocol for the generation, selection and purification of recombinant in vivo–matured Nanobodies for structural biology\textsuperscript{1–5,7,9,19–30} that takes 3–4 months. Our Nanobody discovery platform has the competitive advantage over other recombinant crystallization chaperones\textsuperscript{31–33} that the cloned Nanobody library represents the full collection of the naturally circulating, humoral antigen–binding repertoire of heavy chain–only antibodies, in contrast to combinatorial libraries of conventional antibody fragments. Because Nanobodies are encoded by single exons, the full antigen-binding capacity of in vivo–matured antibodies can be cloned and efficiently screened for high-affinity binders, allowing one to fully exploit the humoral response of large mammals against native antigens. To our knowledge, there are no indications that in vivo–matured Nanobodies induce non-native conformations. Surely, immature B cells expressing antibodies that have to pay a substantial energetic penalty for distorting the antigen structure will have a lower probability of proliferation and differentiation into mature antibody-secreting B lymphocytes.

Limitations

With nearly 20 years of experience we have learned that conformational Nanobodies can be identified against any properly folded protein. In those cases in which we failed in a first attempt, we successfully performed new immunizations or pannings, paying special attention to the quality of the antigen, thereby learning that good protein biochemistry is the key to success. Although Nanobodies are good at binding conformational epitopes on folded proteins with high affinity, they perform poorly at binding...
peptides or intrinsically unfolded parts of proteins. For linear epitopes, conventional antibodies may be a better alternative.

Applications

The Nanobodies to be used as crystallization chaperones can also be valuable for other applications in structural biology. For example, domain-specific Nanobodies have been used in single-particle electron microscopy (EM) as a marker to track these domains in particle projections. Because many Nanobodies can be functionally expressed as intrabodies in eukaryotic cells, these single-domain antibodies can also be used as biosensors to track conformational properties of their targets inside a living cell. Ultimately, Nanobodies that constrain protein targets in unique disease-linked conformations may facilitate the discovery of new therapeutic molecules.

Experimental design

General considerations. The workflow for generating, isolating and characterizing the Nanobodies to be used as crystallization chaperones (Fig. 1) is inherently dependent on the nature of the antigen and on the purpose of the structural study. Several steps in the Nanobody discovery process, including the preparation of the immunogen, the selection strategy, the screening approach and the functional and biophysical characterization, differ when the target is a soluble protein, a membrane protein or a multiprotein complex. To make this protocol broadly applicable for the structural biology community, several modifications to the standard protocol are referred to in the text.

Protein production and antigen quality control. Today, researchers are faced with a bewildering array of methods to produce and purify recombinant proteins. Although this is not the focus of this paper, the supply of properly folded protein is crucial for the generation of conformational Nanobodies. We discourage immunizing animals with poorly characterized protein samples. Our standard protocol typically requires 1 mg of the purified protein: 700 µg for immunization and the remaining portion for all Nanobody selection, identification and characterization efforts. Ideally, a single batch of the purified protein is dispensed into aliquots and stored under conditions that ensure the stability of the protein over time. Often, flash-freezing in liquid nitrogen and storage at −80 °C is favored. We insist on confirming protein quality of one thawed aliquot before the protein is administered as an immunogen. Biochemical or cellular assays that quantitatively assess the functionality of the antigen (e.g., enzymatic and signaling activity, interaction with (radio)ligands or binding of certified conventional antibodies against discontinuous epitopes) can be used to test whether your protein is properly folded. If your protein cannot be tested functionally, we advise performing a rigorous biophysical characterization to confirm its folded state (reviewed in ref. 42). Unless it has been demonstrated not to have any effect on the functionality of the protein, we strongly discourage multiple freeze-thaw cycles in order to minimize protein denaturation. If samples cannot be frozen, consider using freshly prepared protein throughout the immunization and discovery effort.

Conformational locking of the antigens. Nanobodies have been shown to stabilize proteins such as kinases and G protein–coupled receptors (GPCRs) in unique biologically relevant conformations. To identify such Nanobodies, we advise immunizing animals with proteins constrained in the desired conformation with cofactors, enzyme inhibitors, orthosteric ligands, allosteric ligands or any molecule that conformationally traps the macro-molecule in a particular state. We recommend using ligands that dissociate slowly to maximize the lifetime of the constrained target conformation in the immunized animal. Purified, detergent-solubilized membrane proteins may denature after immunization owing to the dissociation of detergents. To maintain a native conformation after immunization, reconstitution of the protein into a lipid environment, typically phospholipid vesicles, or the use of a very tight binding detergent may be required. Reconstitution of membrane proteins in lipid vesicles may also reduce ligand dissociation. A ligand trapped in the vesicle can rebind the intravesicular binding site of the protein, prolonging the extravesicular exposure of the desired conformational state to the immune system. Alternatively, particular mutations may trap the target in a unique conformational state. Finally, chemical cross-linking between protein domains or different proteins may stabilize epitopes that are unique to the complex.

Immunogen preparation, cameld immunization and repertoire cloning. Antibodies with a homodimeric heavy-chain composition devoid of light chains are only found in Tylopo da (camels, dromedaries and llamas) and sharks. Our protocols can be applied to llamas, camels, dromedaries and alpacas. All vaccination experiments are executed according to EU animal welfare legislation and after approval of the local ethics committee. It is known that stress can cause immunosuppression. Animals should be manipulated by authorized staff, preferably by an experienced veterinarian. We allow animals to acclimatize to new housing conditions for at least 1 week before immunization starts.
In llamas, repetitive immunogen administrations generate a robust immune response mediated by both conventional and heavy chain–only antibodies. On the basis of 10 years of experience, GERBU LQ is a good immunostimulating adjuvant for conformational Nanobody discovery, and it is well tolerated by llamas. In general, we inject our animals subcutaneously with cocktails of 1–5 antigens mixed with adjuvant. Alternatively, different immunogens can be injected separately at different s.c. locations. If small-molecule ligands are used for the conformational locking of an antigen, we always add these compounds in excess to the antigen. In our hands, we have successfully reused llamas for different Nanobody discovery projects by respecting a grace period of at least 6 months.

A blood sample of 100 ml from an immunized llama contains sufficient expressing B cells to clone a diverse set of the affinity-matured Nanobodies with high specificity for their cognate antigen. Peripheral blood lymphocytes (PBLs) should be isolated without delay from the noncoagulated blood for the purification of total RNA and the synthesis of cDNA. From this cDNA, the Nanobody-encoding open reading frames can be amplified by PCR and cloned into an appropriate phage display vector.

**Primer design.** Over the years, several PCR strategies have been developed to amplify Nanobody gene fragments from lymphocyte cDNA. We prefer to use a two-step nested PCR approach. One pair of primers (CALL001 and CALL002) has been designed for the first PCR by using the cDNA of B lymphocytes as the template. The CALL002 primer anneals in a region of the second constant heavy-chain domain (CH2) that is conserved among all IgG isotypes of all cameldids, whereas the CALL001 primer anneals in a well-conserved region of the leader signal sequence of all V elements of family III (by far the most abundant V family in cameldids). The primers VHH-Back and VHH-For are used to amplify the Nanobody repertoire via a second nested PCR (Supplementary Fig. 1a). From our experience, the primers described in PROCEDURE Steps 21 and 24 are adequate to amplify Nanobody-encoding genes of family III from dromedary (*Camelus dromedaries*), camel (*Camelus bactrianus*), llama (*Lama glama*), and also alpaca (*Vicugna pacos*). Van der Linden et al. designed dedicated primers annealing to the hinge of each heavy chain–only IgG isotype of all cameldid species (Supplementary Fig. 1b). Maass et al. designed primers dedicated for alpaca Nanobodies (Supplementary Fig. 1c). Kastelic et al. used primers that amplify mixtures of the VHH (variable fragment of the heavy-chain antibody) and the VH (variable fragment of the classical antibody) domains from llama (Supplementary Fig. 1d).

**Selection by phage display.** Many excellent reviews on selection methodologies to enrich for target-specific antibodies against native epitopes have been published. Phage display is certainly the most robust technique, but yeast display or bacterial display can also be used to select Nanobodies from immune libraries. For conformational binders, it is essential to perform the in vitro selection (panning) experiments under conditions (buffer with appropriate detergent, pH, temperature and cofactors) that produce the desired conformation of the protein during phage incubation. To reduce the background of phage-expressing nonspecific Nanobodies, the target protein should be highly pure (>95%) and homogeneous in conformation.

Aspecific adsorption onto the solid surface of an ELISA plate is still the most common way to immobilize targets for selection by phage display, but this method can result in (partial) denaturation of the protein. Although the use of streptavidin-coated magnetic beads is a valid alternative, we perform most panning experiments in 96-well plates, which allows multiple parallel selection conditions. Our preferred method is to capture biotinylated or tagged target protein on a solid phase coated with NeutrAvidin or a tag-specific antibody, respectively. Alternative strategies for presenting target proteins during selection are summarized in Table 1. The structural integrity and homogeneity of the presented target is the most decisive factor for selecting Nanobodies with the desired properties. If possible, we try different capturing or immobilization methods, vary the antigen concentration, use different detergents or ligands to keep the target in the desired conformation, try different washing and incubation buffers or use different elution methods in parallel pannings by using different selection wells on the same 96-well plate. In many cases, magnetic beads can be used as a valid alternative in order to perform selections in solution.

Depending on the magnitude of the heavy chain–only antibody-mediated humoral response in the llama, typically one or two rounds of panning are sufficient to enrich for targetspecific Nanobodies. Rather than selecting for target specificity alone, we prefer implementing conditions early on that allow the identification of Nanobodies with the desired functional or biophysical properties: high affinity, stabilization of a unique protein conformation, modulation of receptor function, binding to a particular target domain or epitope, interference with...
After panning, we routinely Nb8 5. We advise Electrophoretic mobility shift assays on native gels provide a quick and easy strategy for verifying whether purified Nanobodies form a homogeneous complex with the target protein. Figure 2 shows how mobility shift assays have been used to characterize Nanobodies that bind an editosome protein of T. brucei24. Cocrystallization experiments can be successful just by mixing the Nanobody and target protein in a 1.2:1 molar ratio. Alternatively, the complex can be further purified by size-exclusion chromatography after co-incubation. Because Nanobodies are resistant to additives, extremes of pH and temperature, and proteases58, they are ideally suited for screening a broad range of crystallization conditions with variations in pH, ionic strength, temperature, protein concentration, salts, ligands or additives, and type of precipitant. Conditions that can be screened are limited by the stability of the target protein rather than by the stability of the crystallization chaperones. More important is that Nanobodies are extremely soluble proteins (>400 mg ml−1), maximizing the chance that they crystallize in complex with their cocrystallization target rather than yielding Nanobody-only crystals. Because they are efficiently produced in the periplasm of E. coli, SeMet-labeled Nanobodies may ultimately be used for phasing via single-anomalous dispersion technique without the need for introducing SeMet into the target protein58. Alternatively, phase information can be obtained from a molecular replacement solution of the Nanobody in the complex.

Screening and functional/biophysical characterization of conformational Nanobodies. After panning, we routinely pick 96 individual clones from distinct selection outputs and express the Nanobodies in the E. coli periplasm in 96-well plates. In the past 10 years, we have rarely found Nanobodies that bind linear epitopes. Therefore, we discourage the use of western blotting or any other technique that tends to unfold the target. In parallel to the assessment of antigen specificity, assays should be implemented for the identification of Nanobodies with the desired functional or biophysical properties. If the target is a membrane protein, we recommend additionally assessing Nanobody binding to target-overexpressing cells via fluorescence-activated flow cytometry.

Our display vectors permit the inducible periplasmic expression of Nanobodies as soluble C-terminally His6-tagged proteins in E. coli strain WK6. Milligram quantities of >95% pure Nanobody can routinely be obtained by immobilized metal ion affinity chromatography (IMAC) from the periplasmic extract of a 1-liter bacterial culture30.

As it is not known upfront which Nanobodies will behave as the best crystallization chaperones, it is crucial to identify a large panel of sequence-diverse Nanobodies (see Anticipated Results). Typically, after a protein-based immunization and selection discovery, between 3 and 30 Nanobody families are identified. A Nanobody family is defined as a group of Nanobodies with a high similarity in their CDR3 sequence (identical length and >80% sequence identity). Nanobodies from the same family derive from the same B-cell lineage and bind to the same epitope on the target.

Nanobodies as crystallization chaperones. Electrophoretic mobility shift assays on native gels provide a quick and easy strategy for verifying whether purified Nanobodies form a homogeneous complex with the target protein. Figure 2 shows how mobility shift assays have been used to characterize Nanobodies that bind an editosome protein of T. brucei24. Cocrystallization experiments can be successful just by mixing the Nanobody and target protein in a 1.2:1 molar ratio. Alternatively, the complex can be further purified by size-exclusion chromatography after co-incubation. Because Nanobodies are resistant to additives, extremes of pH and temperature, and proteases58, they are ideally suited for screening a broad range of crystallization conditions with variations in pH, ionic strength, temperature, protein concentration, salts, ligands or additives, and type of precipitant. Conditions that can be screened are limited by the stability of the target protein rather than by the stability of the crystallization chaperones. More important is that Nanobodies are extremely soluble proteins (>400 mg ml−1), maximizing the chance that they crystallize in complex with their cocrystallization target rather than yielding Nanobody-only crystals. Because they are efficiently produced in the periplasm of E. coli, SeMet-labeled Nanobodies may ultimately be used for phasing via single-anomalous dispersion technique without the need for introducing SeMet into the target protein58. Alternatively, phase information can be obtained from a molecular replacement solution of the Nanobody in the complex.

Considerations about the laboratory facilities. We advise dedicating two separate laboratories for Nanobody discovery. One phage-free laboratory is used for Nanobody repertoire amplification and cloning. We use dedicated reagents for library construction. The other laboratory is used for all phage work, including the amplification of phage and pannings. To reduce phage contamination, we use filter tips and clean benches, equipment and glassware with 1% (wt/vol) sodium hypochlorite after each experiment. Use disposables whenever possible, and discard them in 1% (wt/vol) sodium hypochlorite to inactivate the remaining phage particles.

**MATERIALS**

**REAGENTS**

- **Llamas**: CAUTION All vaccination experiments should be executed in accordance with the applicable animal welfare legislation, and they must be approved by the local ethics committee.
- Acetic acid, glacial (Merck, cat. no. 1.00058.2500) CAUTION Glacial acetic acid is corrosive. Avoid inhalation and exposure to skin and eyes.
- AEBSF protease inhibitor (Carl Roth, cat. no. 2931.3)
- Agarose MP (Roche, cat. no. 11 388 991 001)
- AP-conjugate substrate: 4-nitrophenyl phosphate disodium salt hexahydrate (DNPP, Sigma-Aldrich, cat. no. 71768)
- CaCl₂·2H₂O (Merck, cat. no. 2382)
- Chloroform (Merck, cat. no. 1.02445.0250)
- Citric acid·H₂O (Sigma-Aldrich, cat. no. C1909)
- E. coli strains CRITICAL Use an amber codon suppression (supE) strain such as TG1 (electrocompetent cells 24 x 10¹⁰ c.f.u. µg⁻¹; Lucigen, cat. no. 60502-1) for cloning and preparation of phage libraries,
**PROTOCOL**

and a nonsuppressor strain (su−) such as WK6 (ref. 59) for the expression of Nanobodies
• EDTA (Sigma-Aldrich, cat. no. E5134)
• ELISA-blocking reagent: skimmed milk powder (any commercial provider, e.g. Nestle, Marvell)
• Ethanol (Fischer Chemical, cat. no. E060DF/15)
• GERBU adjuvant LQ 3000 (GERBU Biotechnik, cat. no. 30000025)
• Glycerol (VWR, cat. no. 24387-292)
• Guanidine-HSCN (ICN Biomedicals, cat. no. 820991)
• HRP-conjugate substrate: azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid)
• (ABTS, Sigma-Aldrich, cat. no. A1888)
• IPTG, dioxane-free (Thermo Scientific, cat. no. R0392) for the induction of Nanobody expression
• Isoamyl alcohol (Acros Organics, cat. no. 41273-5000)
• KAPA Taq DNA polymerase and 10× Taq buffer A (KAPA Biosystems, cat. no. BK10012) and dNTP mix (Thermo Scientific, cat. no. R0192)
for PCRs
• KCl (VWR, cat. no. 26764-298)
• KH₂PO₄ (Carl Roth, cat. no. 3904.1)
• K₂HPO₄ (Carl Roth, cat. no. 749.2)
• M13 helper phage: kanamycin-resistant VCSM13 (Stratagene, cat. no. 200251)
• MgCl₂ (Sigma-Aldrich, cat. no. M8266)
• MgSO₄·7H₂O (Merck, cat. no. 5889)
• NaCl (Carl Roth, cat. no. 3957.1)
• NaH₂PO₄·H₂O (Merck, cat. no. 1.06586.1000)
• Na₂HPO₄·H₂O (Carl Roth, cat. no. K300.1)
• NH₄Cl (Sigma-Aldrich, cat. no. A9434)
• NaHCO₃ (Merck, cat. no. 6329)
• NeutrAvidin biotin-binding protein (Thermo Scientific, cat. no. 31000)
• PEG6000 (Sigma-Aldrich, cat. no. 81260)
• Phage display vectors: pMES4 (GenBank GQ907248) and pMESy4 (GenBank KH415192) are pHEN4 (ref. 60) derivatives allowing the display and production of soluble His₆-tagged (pMES4) or His₆-tagged and EPEA-tagged* (i.e., CaptureSelect C-tag) Nanobodies (pMESy4), respectively. See Supplementary Figures 2 and 3 for maps. Nanobodies are under the transcriptional control of the lacZ promoter that is repressed by glucoside and induced by IPTG
• RNaseOUT recombinant RNase inhibitor (Life Technologies, cat. no. 10777019)
• Sodium acetate-3H₂O (Merck, cat. no. 1.06267.1000)
• Sodium hypochlorite (any supplier of commercial bleach) **CAUTION** Avoid inhalation and exposure to skin and eyes.
• Sucrose (Sigma-Aldrich, cat. no. S7903)
• SuperScript II reverse transcriptase (SSII reverse transcriptase; Thermo Scientific, cat. no. EL0011)
• T4 DNA ligase, 1 U (Thermo Scientific, cat. no. E10606)
• Trizma (Tris) base (Sigma-Aldrich, cat. no. T6066)
• Tri-sodium citrate-2H₂O (Sigma-Aldrich, cat. no. W302600)
• Trypsin (Sigma-Aldrich, cat. no. T1426) for the elution of phage
• Ultrapure phenol:water (3.75:1 (vol/vol)) (Life Technologies, cat. no. 15594-047)
• Yeast extract (Duchefa, cat. no. Y1333)
• Thiamine hydrochloride (Sigma-Aldrich, cat. no. T1270)
• Yeast extract (Duchefa, cat. no. Y1333)
• **Restriction enzymes**
• Eco911 (Thermo Scientific, cat. no. ER0391)
• Ncol (Thermo Scientific, cat. no. ER0571)
• PstI (Thermo Scientific, cat. no. ER0611)
• XbaI (Thermo Scientific, cat. no. ER0681)
• DNA purification kits
• PureYield plasmid miniprep system (Promega, cat. no. A1222)
• QIAquick gel extraction kit (Qiagen, cat. no. 28706)
• QIAquick PCR purification kit (Qiagen, cat. no. 28106)
• Biotinylation kits
• EZ-Link Sulfo-N-hydroxysulfosuccinimide (NHS)-LC-biotinylation kit (Thermo Scientific, cat. no. 21435)
• Biotin quantitation kit (Thermo Scientific, cat. no. 28005)
• **Antibodies**
• Capture select biotin anti-C-tag conjugate (Life Technologies, cat. no. 7103252100)
• Goat anti-labna antibody, HRP-conjugated (Bethyl Laboratories, cat. no. A160)
• Goat anti-mouse IgG, alkaline phosphatase–conjugated (Sigma-Aldrich, cat. no. A3562)
• Mouse anti-His antibody (Abd Serotec, cat. no. MCA1396)
• **Antibiotics**
• Ampicillin sodium salt (Carl Roth, cat. no. K029.2)
• Kanamycin monosulfate (Duchefa, cat. no. K0126)
• **Detergents**
• Laureyl sarcosine (Affymetrix, cat. no. 21633)
• Tween20 (Sigma-Aldrich, cat. no. P2287 or P1379)
• **Primers**
• CALL001: 5′-GTCTGCGTGTAGACTGGTC-3′; CALL002: 5′-GGTGACGTTGCTGATACCTGG-3′; CALL003: 5′-CATGTCGCGCGCCGCTG-3′ (Eco911); MP57: 5′-TTATGCTTCCGGGCTGTATG-3′; GII: 5′-CCACAGACGGGTCATAGAC-3′. These custom-synthesized primers (Sigma-Aldrich) are stored at −20 °C for years as stock solutions at 100 µM in 10 mM Tris-HCl buffer (pH 8.5)
• dN6 random primers (5′-NNNNNN-3′) are custom-synthesized and stored at −20° for years as a stock solution in 10 mM Tris-HCl buffer (pH 8.5) at a 2.5 µg µl⁻¹ concentration
• **Equipment**
• Benchtop centrifuge for Falcon tubes and 96-well plates, swing-out rotor (Eppendorf, cat. no. 5810R)
• Blood collection tubes: Venosafe hematolog (Terumo, cat. no. VF-109SDK) and serum gel (Terumo, cat. no. VF-108SAS) tubes
• Cell scraper (Costar, cat. no. 3011)
• Conical-bottom plates, 96 wells (Thermo Scientific, cat. no. 249944)
• Centrifuge for 1.5- and 2-ml tubes (Eppendorf, cat. no. 5430R)
• Electroporator: E.coli pulser (Bio-Rad, cat. no. 1652103)
• Falcon conical centrifuge tubes (15 ml and 50 ml) (Fisher Scientific, cat. no. 14-959-49B and 14-432-22)
• Gene pulser electroporation cuvette, 0.1 cm gap (Bio-Rad, cat. no. 165-2089)
• Glass beads, diameter 2.5–3.5 mm (VWR, cat. no. 2010807)
• Heidolph Titramax 1000 vibrating platform (Heidolph Instruments, cat. no. 544-12200-00)
• Incubators at 4 °C, 16 °C and 37 °C
• Incubators with orbital shaking platform at 37 °C, 170–200 r.p.m.
• Maxisorp 96-well Immunoplates (Nunc, cat. no. 439454)
• Multichannel pipettes: Finnpipette F2 eight channels, 30–300 µl (Thermo Scientific, cat. no. 4662030); Finnpipette F2 eight channels, 5–50 µl (Thermo Scientific, cat. no. 4662010)
• Nunc microplate sealing tape 236366 (Thermo Scientific, cat. no. 10170671)
• PCR tube strips (8-tube strips, Greiner Bio-One, cat. no. 673210; cap strips, Greiner Bio-One, cat. no. 373270)
• Plates, 96 deep wells (Thermo Scientific, cat. no. AB-0932)
• RNase-free microtubes (Eppendorf, cat. no. 0030 121.589)
• Round-bottom tissue culture plates with lid, 96 wells (BD Biosciences, cat. no. 353077)
• Shake flask, triple baffled, 1 liter, 250 ml (Sigma-Aldrich, cat. nos. F0769 and F0644)
• Spectrophotometer and ELISA plate reader
• Square dishes, 245 × 245 × 25 mm (Thermo Scientific, cat. no. 240835)
• Sterile gas-permeable adhesive seal (Thermo Scientific, cat. no. AB-0932)
• Sterile siliconized glass vials (Thermo Scientific, cat. no. 301160)
• Sterile sodium hypochlorite (any supplier of commercial bleach)
• Sterile gas-permeable adhesive seal (Thermo Scientific, cat. no. AB-0932)
• Sterile siliconized glass vials (Thermo Scientific, cat. no. 301160)
• **Reagent setup**
• **Glucose, 20% (wt/vol)** Dissolve 20 g of glucose in 100 ml of ddH₂O by heating. Autoclave the solution and store it at room temperature (RT = 22 °C) for months.
• **PEG6000 (20% (wt/vol))/2.5 M NaCl solution** Dissolve 200 g of PEG6000 and 146.1 g of NaCl in 1 liter of ddH₂O. Autoclave the solution and store it at 4 °C. ▲ CRITICAL Dissolving PEG and NaCl requires stirring for several hours.
**PROTOCOL**

**PROCEDURE**

**Protein storage and analysis ● TIMING 1 d**

1. Dispense ~1 mg of protein in seven aliquots of 100 μg (for llama immunization) and store the remaining amount of protein in 10- to 20-μg aliquots. Use PCR tubes and flash-freeze the aliquots by submerging the tubes in liquid nitrogen⁶², and then transfer them for long-term storage to −80 °C.

2. Dispense ten 5-ml aliquots of the protein storage buffer (storage buffer composition has to be optimized for each target protein; see ref. 42) and store them under appropriate conditions. For each component of the storage buffer, consult the manufacturer’s product sheets.

3. Thaw one protein aliquot rapidly in your hand⁶² and confirm that the protein is properly folded by using at least one quantitative functional or biophysical assay⁶².  

   **CAUTION** Repeated freeze-thaw cycles may negatively affect protein quality.

**Llama immunization ● TIMING 6 weeks**

4. We routinely immunize llamas with mixtures of five different target proteins. Always thaw protein aliquots (from Step 1) rapidly in your hand⁶² and keep them on ice. For the first immunization, mix 200 μg of each protein, and for the following immunizations mix 100 μg of each protein in a buffer with a composition that does not disrupt the integrity of any of the proteins in the mix. Keep the total volume between 300 μl and 1 ml. If proteins cannot be mixed, separate immunogens can
be prepared to be injected separately at different s.c. locations near draining lymph nodes. Table 2 gives a non-exhaustive
overview of alternative materials that can be used for immunization.

**CAUTION** All vaccination experiments should be executed in accordance with the applicable animal welfare legislation,
and they must be approved by the local ethics committee. Make sure not to inject additives such as sodium azide or
(therapeutic) compounds that are toxic to the animals.

5| Before the first immunogen administration, collect 4 ml of blood sample in a Venosafe serum gel tube and allow it to
clot for 2 h at RT. Centrifuge the sample for 5 min at RT and 3,000 \( \times \) g and recover the supernatant as the preimmune serum.
Store the serum at −20 °C for measuring the serum conversion (Step 11).

6| Immunize the animals six times with the freshly prepared immunogen (from Step 4) at weekly intervals. For the
first, second, third and fifth immunizations, gently mix the immunogen with an equal volume of the GERBU adjuvant to
make an emulsion, and inject it subcutaneously (maximum 2 ml) in the neck base of the llama near the bow lymph node.
To eliminate the risk of the adjuvant denaturating your protein of interest and destroying its conformational epitopes, we
advise not to mix the immunogen with the adjuvant for the fourth and the sixth immunizations. Rather, inject the native
immunogen subcutaneously and separately inject an equal volume of GERBU at a 5-cm distance from the immunogen to
locally boost the immune system.

**Blood sampling and lymphocyte preparation** ● **TIMING 1 d**

7| Three to five days after the last immunization, collect 100 ml of blood from the jugular vein in Venosafe hematology
EDTA-coated blood collection tubes and invert them twice to inhibit coagulation. Transfer the blood samples to the lab and
process them immediately according to Step 8.

▲ **CRITICAL STEP** Coagulated blood will impair the extraction of total RNA from the lymphocytes.

8| Equally distribute the nondiluted blood into four separate Uni-Sep\textsubscript{MAXI} tubes and isolate the PBLs according to the
manufacturer’s instructions. Collect PBLs at the interface between the plasma and polysucrose–sodium metrizoate layers, and
transfer them to four separate 50-ml Falcon tubes. Recover the plasma (top layer) as the postimmune IgG-containing sample,
which will be used to measure the serum conversion in Step 11. Plasma samples can be stored at −20 °C for years without
notable loss of activity.

9| Dilute the PBLs at least tenfold in PBS and centrifuge the tubes for 20 min at 800 g and 4 °C.

10| Carefully discard the supernatants. Resuspend each cell pellet in at least 5 ml of PBS, collect the cell suspensions into one
50-ml Falcon tube and then divide the cells equally over two 50-ml Falcon tubes. Centrifuge the cells as in Step 9, discard
the supernatant and place the tubes upside down for a few minutes on a paper towel. Each pellet contains ~5 \( \times \) 10\(^8\) PBLs.
PREFERABLY, continue immediately with the extraction of total RNA from fresh PBLs (Step 13) without freezing. RNA can also
be extracted from pellets that have been stored at −80 °C for periods shorter than 6 months.

**Analysis of serum conversion** ● **TIMING 2 d**

11| Use ELISA to compare the titer of the preimmune serum (from Step 5) and the postimmune plasma (from Step 8)
to measure the serum conversion induced by each antigen (as described in Box 1). Make fourfold serial dilutions of

---

**TABLE 2** | Immunogen formats that have been applied for the generation of conformational Nanobodies.

<table>
<thead>
<tr>
<th>Format</th>
<th>Notes/Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergent-solubilized membrane proteins</td>
<td>can be mixed with other soluble proteins if these resist the detergent(^{26}).</td>
</tr>
</tbody>
</table>
| Reconstituted membrane proteins             | membrane proteins tend to be more stable when they are reconstituted in lipid vesicles.
|                                             | We successfully immunized llamas with GPCRs, reconstituted in DOPC:lipid A (10:1) vesicles\(^1\) as exemplified in the Anticipated Results. |
| Protein complexes                           | if (transient) protein complexes are immunized, we advise chemically cross-linking the complex. In general, we use a commercial mixture of different amine reactive cross-linkers that have been developed for mass spectroscopic detection of protein-protein complexes\(^7\). |
| Intact cells or membrane preparations      | membrane preparations functionally expressing the target protein are described\(^{17,43}\). |
| Genetic immunization                        | llamas have been immunized successfully with a DNA-prime protein-boost strategy\(^{16,17}\).|

---
Box 1 | ELISA ● TIMING 2 d

Unless otherwise mentioned, all incubation steps are performed at RT on a vibrating shaking platform (700 r.p.m.) or at any temperature that is required to keep the protein stable.

1. Add 100 µl of a 2–5 µg ml⁻¹ NeutrAvidin solution in PBS to individual wells of a 96-well Maxisorp plate. Incubate the plate overnight at 4 °C. Wash the wells three times with 250 µl of PBST. Add 250 µl of blocking solution to all wells and block the plate for 2 h. Wash it 5 times with 250 µl of PBST.

2. Add 100 µl of the biotinylated target protein (diluted to 1 µg ml⁻¹ in the protein storage buffer) per well. Include appropriate negative controls in separate wells (e.g. irrelevant biotinylated protein) and incubate for 30 min. Wash it five times with 250 µl of the protein storage buffer.

3. Add 100 µl of the diluted sample in protein storage buffer containing 0.1% (wt/vol) skimmed milk powder to each well and incubate for 1 h. Wash five times with 250 µl of the protein storage buffer. For serum conversion assays, use serial dilutions of the preimmune and postimmune sera as described in Step 11. For Nanobody screening, use a fivefold dilution of periplasmic extracts as described in Step 50.

4. If required, add secondary antibody in 100 µl of protein storage buffer containing 0.1% (wt/vol) milk and incubate for 1 h. Wash it five times with 250 µl of the protein storage buffer.

5. Add the conjugate (enzyme-labeled anti-immunoglobulin) in 100 µl of the protein storage buffer containing 0.1% (wt/vol) milk and incubate for 1 h. Wash it five times with 250 µl of the protein storage buffer.

6. Develop the reaction by adding 100 µl of the appropriate colorimetric substrate (ABTS or DNPP reagent), and measure the absorption at an appropriate wavelength.

| Isolation of total RNA ● TIMING 1 d |

CAUTION For all manipulations of RNA, wear gloves and use RNase-free materials and reagents.

13| Add 4 ml of PBL lysis buffer to ~5 × 10⁸ PBLs (from Step 10), lyse the cells and shear the genomic DNA by repeatedly forcing the solution through 19-G and 23-G needles (six times each). Transfer the transparent solution to a 15-ml Falcon tube.

14| Add 400 µl of 2 M sodium acetate (pH 4.0), 4 ml of phenol (water saturated) and 2 ml of a 24:1 chloroform:isoamyl alcohol solution. Mix well without vortexing, and then incubate the mixture for 10 min on ice. Centrifuge the mixture for 10 min at 3,200 g and 4 °C in a swing-out rotor.

15| Carefully transfer the upper RNA-containing water phase to a fresh 15-ml Falcon tube and avoid carrying over interphase material. Add 4 ml of phenol (water saturated) and 2 ml of chloroform/isoamyl alcohol (24:1) solution. Mix well and incubate the mixture for 10 min on ice. Centrifuge the mixture for 10 min at 3,200g and 4 °C in a swing-out rotor.

16| Transfer 3 ml of the upper phase to a fresh 15-ml Falcon tube and divide it over six RNase-free microcentrifuge tubes. Add 500 µl of ice-cold 100% ethanol to each tube, mix it manually and store it at −80 °C for at least 30 min (or overnight) to precipitate the nucleic acids.

17| Centrifuge the tubes for 20 min at 20,000g and 4 °C. Next, discard the supernatants and air-dry the pellets for 10 min at RT. Dissolve each pellet in 20 µl of RNase-free water. Incubate the pellets for 5 min at RT. If the pellets do not dissolve completely, incubate the RNA pellets for 10 min at 65 °C. Collect all six samples in a single RNase-free microcentrifuge tube.

18| Quantify RNA by measuring the OD at 260 nm. Typically, 100–250 µg of RNA is obtained from 50 ml of blood.

PAUSE POINT For long-term storage at −80 °C, the RNA must be precipitated by mixing 0.05 volumes of 2 M sodium acetate (pH 4.0) and 2.5 volumes of 100% ethanol. Precipitated RNA can be stored at −80 °C for 1 year without detectable degradation.
cDNA synthesis ● TIMING 4 h
19] To prepare for cDNA synthesis, dilute 50 µg of total RNA in 50 µl of RNase-free water in a PCR tube. Add 2.5 µg of dN6 random primers, incubate for 7 min at 65 °C and allow the primers to anneal for 5 min on ice. Precipitate the remaining RNA (as described in Step 18) for later use.

20] To synthesize cDNA, add the following components to the PCR tube: 20 µl of 5× SSII reverse transcriptase buffer, 10 µl of 100 mM DTT, 2 µl of dNTPs (25 mM), 2 µl of RNaseOUT, 2.5 µl of SSII reverse transcriptase and 12.5 µl of RNase-free water. Incubate the mixture for 10 min at 25 °C, 2 h at 42 °C and 15 min at 72 °C.

■ PAUSE POINT cDNA can be stored at −80 °C for years without losing the diversity of the antibody repertoire.

Construction of the immune library ● TIMING 5 d
21] To amplify the variable domains of all immunoglobulin heavy chains (VHs and VHHs) from the cDNA (Supplementary Fig. 1a) with two gene-specific primers CALL001 and CALL002 (see Primer Design), combine the following components in a PCR tube:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>cDNA</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>KAPA Taq buffer A, 10×</td>
<td>1×</td>
</tr>
<tr>
<td>2.0</td>
<td>CALL001 primer (10 µM)</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>2.0</td>
<td>CALL002 primer (10 µM)</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>1.0</td>
<td>dNTPs (10 mM each)</td>
<td>200 µM</td>
</tr>
<tr>
<td>38.75</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>KAPA Taq DNA polymerase (5 U µl⁻¹)</td>
<td>0.025 U µl⁻¹</td>
</tr>
</tbody>
</table>

Amplify DNA in a thermocycler with the following amplification cycles:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>3 min</td>
</tr>
<tr>
<td>2–31</td>
<td>94</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>1 min</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>45 s + 0.02 min per cycle</td>
</tr>
<tr>
<td>32</td>
<td>72</td>
<td>7 min</td>
</tr>
</tbody>
</table>

▲ CRITICAL STEP We recommend running several PCRs in parallel, starting with 0, 0.5, 1, 2 and 4 µl of cDNA template.

22] Analyze the PCR products by electrophoresis on a 1% (wt/vol) agarose gel. Two DNA fragments should be amplified: fragments of ~700 bp representing the heavy chain–only antibody repertoire and fragments of 1,000 bp corresponding to the heavy chain of the conventional antibodies (Supplementary Fig. 1a). Choose the condition in which an intense 700-bp band is well separated from the background and repeat this PCR in eight tubes.

▲ TROUBLESHOOTING

23] To separate and gel-purify the 700-bp PCR fragment from the 1,000-bp fragment, apply the PCR products to eight lanes on a 1% (wt/vol) TBE agarose gel. Run the gel at 5.5 V cm⁻¹ for 35 min. Cut the 700-bp PCR products from the gel and purify the fragment cut from each lane separately by using the QIAquick gel extraction kit according to the manufacturer’s instructions. Quantify the DNA from each purification by measuring the OD₂₆₀.

■ PAUSE POINT The gel-extracted and purified PCR products can be stored at −20 °C for weeks without losing the diversity of the antibody repertoire.
Re-amplify the Nanobody-encoding genes with nested primers VHH-Back and VHH-For, annealing at framework 1 and framework 4, respectively (Supplementary Fig. 1a), by combining the following components in eight PCR tubes:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>Gel-purified PCR product (from Step 23)</td>
<td>0.4 ng µl⁻¹</td>
</tr>
<tr>
<td>5.0</td>
<td>KAPA Taq buffer A, 10×</td>
<td>1 ×</td>
</tr>
<tr>
<td>2.0</td>
<td>VHH-Back primer (10 µM)</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>2.0</td>
<td>VHH-For primer (10 µM)</td>
<td>0.4 µM</td>
</tr>
<tr>
<td>1.0</td>
<td>dNTPs (10 mM each)</td>
<td>200 µM</td>
</tr>
<tr>
<td>34.75</td>
<td>Water</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>KAPA Taq DNA polymerase (5 U µl⁻¹)</td>
<td>0.025 U µl⁻¹</td>
</tr>
</tbody>
</table>

Amplify the DNA in a thermocycler with the following amplification cycles:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94</td>
<td>5 min</td>
</tr>
<tr>
<td>2–16</td>
<td>94</td>
<td>30 s</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>30 s</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>30 s</td>
</tr>
<tr>
<td>17</td>
<td>72</td>
<td>7 min</td>
</tr>
</tbody>
</table>

Clean up each PCR reaction with the Qiagen PCR purification kit, according to the manufacturer’s instructions, and then pool all PCR products in one microcentrifuge tube.

Follow the restriction enzyme manufacturer’s instructions to digest 10 µg of the phage display vector pMES4 or pMESy4 with PstI, Eco91I and XbaI in a 250-µl reaction mix. The additional XbaI digestion reduces self-ligation of the vector. In parallel, digest 3 µg of the amplified PCR product (from Step 25) with PstI and Eco91I in a 100-µl reaction. Purify the vector and the PCR product by using the QIAquick PCR purification kit, according to the manufacturer’s instructions.

Pause Point Store the digested phage display vector and PCR product at −20 °C. They can be stored for a minimum of 6 months without loss of cloning efficiency.

Carry out test ligations to determine the amount of vector and insert needed to generate a library of at least 10⁷ individual transformants by ligating 100 ng of the triple-digested vector with 30 ng of the PstI/Eco91I-digested PCR product (1:3 molar ratio) and by ligating 100 ng of the triple-digested vector alone for 2 h at RT in a 10-µl reaction by using 0.5 units of the ligase.

Purify these ligation mixtures with the QIAquick PCR purification kit, according to the manufacturer’s instructions, and transform 5 µl of each into 25 µl of electrocompetent TG1 E. coli cells by electroporation according to the manufacturer’s instructions.

Make tenfold serial dilutions of the recovered transformed cells in LB. Plate 100 µl of the 1:10,000, 1:1,000 and 1:100 dilutions on 90-mm LB agar plates containing 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose and allow it to grow overnight at 37 °C.

Count the colonies on each plate. Multiply the number of colonies with the corresponding dilution factor to calculate the electroporation efficiency. Pick 20 separate colonies and resuspend each colony in 50 µl of ddH₂O for colony PCR with primers MP57 and GIII (as described in Step 68). Analyze the PCR products (Step 69) to confirm that the majority (>75%) of the transformants has an insert of the proper size of a Nanobody.
**PROTOCOL**

**● CRITICAL STEP** If the electroporation efficiency per 100-ng vector in the vector-insert ligation mix is <5 x 10^2, or if <75% of the transformants have a Nanobody insert, we advise you to repeat Steps 26–30.

**? TROUBLESHOOTING**

31] Ligate sufficient digested vector and insert prepared in Step 26 to generate a library of at least 10^7 individual transformants. We typically ligate 15–25 times the amount of the test ligation overnight at 16 °C.

32] Purify the ligation mixture by using the QIAquick PCR purification kit on a single column, wash it twice with PE solution and elute with 30 µl of EB buffer (included in the kit).

33] Perform five separate electroporations each by using 6 µl of the purified ligation and 25 µl of TG1 cells. To each cuvette, add 1 ml of prewarmed recovery medium (37 °C) and recover all cells in one 50-ml Falcon tube; incubate the cells for 1 h at 37 °C and 170 r.p.m. Add LB to reach a total volume of 8 ml.

34] Use 100 µl of the electroporated cells to make serial dilutions (Step 29) and calculate the effective library size (Step 30). Analyze the final insert ratio according to Step 30.

35] Divide the remaining suspension over four 245-mm square LB-agar dishes containing 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose and grow overnight at 37 °C.

36] When the library size is ≥10^7, recover the library; wet the 245-mm square LB-agar dishes with 4 ml of LB, scrape with a sterile cell scraper and collect all cells in a 50-ml Falcon tube with a sterile pipette. Rinse the plates once more, each with 2 ml of LB medium, and mix the collected cell suspension with sterile glycerol (20% (vol/vol) final concentration). Measure the OD_{600} of the suspension, make 10–20 aliquots of 150 µl and store them at −80 °C. The remaining suspension can be stored in larger tubes at −80 °C.

**PAUSE POINT** These glycerol stocks of the immune library can be stored at −80 °C for several years without notable loss of repertoire diversity. Immune libraries can be used as the starting point to select Nanobodies each time new reagents or new scientific insights become available.

**Rescue and amplification of phage from immune libraries ● TIMING 2 d**

37] **Day 1.** Before each round of panning, rescue and amplify Nanobody-displaying phage particles by adding helper phage according to the standard rescue protocol: first, inoculate six OD_{600} units of the immune library into a baffled 250-ml Erlenmeyer containing 60 ml of 2× TY medium supplemented with 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose. For a typical library size of 10^8 clones, this inoculum represents 48 copies of each library clone (assuming one OD_{600} unit of *E. coli* cells corresponds to 8 x 10^8 cells per ml) and theoretically 480 copies of the maximum Nanobody diversity present in a 100-ml blood sample (estimated to be maximally 10^7). Grow the cells at 37 °C and 200 r.p.m. until the cells reach logarithmic phase (corresponding to an OD_{600} of 0.5) required for F-pilus production.

**● CRITICAL STEP** Note that phage display libraries can only be rescued from pMES4 or pMESy4 in an amber codon suppressor strain such as TG1 that contains the F’ conjugative plasmid.

38] For phage rescue, transfer 10 ml of the log-grown cells to a sterile 50-ml Falcon tube. Superinfect the cells with 4 x 10^{10} p.f.u. VCSM13 (10 times excess helper phage versus TG1 cells). Mix gently and incubate the cells for 30 min at 37 °C without shaking, allowing the phage to infect the cells.

**● CAUTION** As TG1 cells are easily contaminated with phage, always use filter tips when you are working with phage to avoid unintended TG1 infections, and discard all tubes containing phage in 1% (wt/vol) sodium hypochlorite to inactivate remaining phage particles.

39] Centrifuge the infected cells at 2,800g for 10 min at RT, and discard the supernatant carefully to remove traces of glucose. Resuspend the cell pellet in 50 ml of 2× TY supplemented with 100 µg ml⁻¹ ampicillin and 25 µg ml⁻¹ kanamycin into a baffled 250-ml Erlenmeyer flask. Incubate overnight at 37 °C and 200 r.p.m. for the amplification of Nanobody-displaying phage.

40] **Day 2.** Transfer the overnight culture to a 50-ml Falcon tube and centrifuge it for 15 min at 3,200g and 4 °C in a swing-out centrifuge to pellet the cells.

41] Transfer 40 ml of the supernatant to a new 50-ml Falcon tube, and add 10 ml of 20% (wt/vol) PEG6000/2.5 M NaCl solution. Mix well by inverting the Falcon tube five times, and then keep the tube on ice for at least 30 min to precipitate the phage, resulting in a homogeneous cloudy suspension.
42| Pellet the phage particles by centrifugation for 10 min at 3,200g and 4 °C. Discard the supernatant and carefully remove the remaining liquid by placing the tube upside down on tissue paper.

43| Resuspend the precipitated phage particles in 1 ml of ice-cold PBS, and transfer the suspension to a microcentrifuge tube. Centrifuge the tube for 1 min at 20,000g and 4 °C in a microcentrifuge to pellet residual bacteria.

44| Transfer the supernatant to a new microcentrifuge tube without disturbing the bacterial pellet, and re-precipitate the phage by adding 250 µl of ice-cold 20% (wt/vol) PEG6000/2.5 M NaCl solution. Invert the tube 5–10 times until a homogeneous white suspension appears, and then keep the tube on ice for 10 min.

45| Centrifuge the tube for 15 min at 20,000g and 4 °C in a microcentrifuge, remove the supernatant and resuspend the phage pellet in 1 ml of ice-cold PBS. Centrifuge for 1 min at 20,000g and 4 °C in a microcentrifuge to completely remove bacterial contaminants. Transfer the supernatant to a new tube to recover the phage solution.

■ PAUSE POINT The phage is ready to be used for panning, and it can be stored for up to a month at 4 °C without loss of phage infectivity. For longer-term storage, add glycerol (20% (vol/vol) final concentration) and transfer it to −80 °C. Before use, remove glycerol from the solution by precipitating the phage as described in Steps 44 and 45.

Antigen presentation and phage selection by panning ● TIMING 3 d per round of selection

46| Day 1: preparative steps. Inoculate a fresh colony of TG1 cells grown on minimal medium into 15 ml of LB in a 100-ml culture flask and incubate it at 37 °C and 170 r.p.m. Grow the cells until they are in the exponential phase (OD$_{600}$ = 0.5–0.6) and keep them sterile on ice.

47| To estimate the number of infective recombinant phage, titrate the rescued phage (from Step 45) by preparing serial tenfold dilutions in a low-binding 96-well round-bottom culture plate. Typically, prepare up to 10$^{10}$-fold dilutions by sequentially mixing 10 µl of the phage into 90 µl of PBS. Transfer 10 µl of each dilution to a well with 90 µl of TG1 cells (from Step 46). Use multichannel pipettes when possible. Incubate the plate for 15 min at 37 °C without shaking to infect TG1 cells. Carefully pipette 5-µl drops of the infected TG1 cells (one drop per phage dilution) on solid selective medium (LB + 100 µg ml$^{-1}$ ampicillin + 2% glucose), air-dry the drops and grow them overnight at 37 °C.

48| As TG1 cells are easily contaminated with phage, also pipette a 5-µl drop of the noninfected TG1 culture (from Step 46) on solid selective medium as a negative control.

49| Coat nonadjacent wells of a Maxisorp 96-well plate with 100 µl of a 2 µg ml$^{-1}$ NeutrAvidin solution in PBS and seal the plate with microplate sealing tape. Incubate overnight at 4 °C. Sealed plates can be stored for 2 weeks at 4 °C without loss of capturing capacity.

50| Day 2: enriching for target-specific recombinant phage. Estimate the phage titer by counting the colonies from the highest dilutions, prepared in Step 47, that have grown overnight onto the selective medium.

▲ CRITICAL STEP If the titer of the phage library is lower than 10$^{12}$ colony-forming units (c.f.u.) per ml, we advise repeating the library rescue (Steps 37–48).

? TROUBLESHOOTING

51| Make sure that the log-grown TG1 culture (from Step 48) was not infected with phage by visually inspecting the overnight-incubated solid medium plate. No TG1 colonies should be grown on selective medium.

52| Dilute the TG1 culture (from Step 46) 100-fold into 50 ml of LB and incubate it at 37 °C and 170 r.p.m. Grow the cells until they are in the exponential phase (OD$_{600}$ = 0.5–0.6), and keep them on ice until used in Steps 59 and 60.

53| Wash the NeutrAvidin-coated wells (from Step 49) three times with 250 µl of PBST, add 250 µl of blocking solution and incubate the plate for 2 h on a vibrating platform (700 r.p.m.) at RT.

54| For each selection well and each control well, preincubate 10$^{11}$ phage (from Step 45) with 10 µl of blocking solution in 100 µl of the appropriate selection buffer for 30 min at RT in one microcentrifuge tube by head-over-head rotation. In our hands, the presence of trace amounts of biotin in milk powder does not affect antigen capturing.
55| Biotinylate the target protein for panning by using the Thermo Scientific EZ-Link sulfo-NHS-LC-biotinylation kit, according to the manufacturer’s instructions.

▲ CRITICAL STEP Although it is beyond the scope of this protocol, it is important to quantify the biotin/target ratio (ideally 1) with the biotin quantitation kit and to demonstrate that biotinylation does not interfere with the quality of the target.

56| To immobilize the biotinylated target protein for panning, remove the blocking solution of the NeutrAvidin-coated wells (from Step 53) and wash five times with 250 µl of the appropriate selection buffer. For each selection condition, add saturating amounts of the biotinylated target protein (from Step 55; typically 100 nM) in 90 µl of the appropriate selection buffer supplemented with 10 µl of blocking solution to a separate selection well. For each selection condition, also fill a NeutrAvidin-coated control well with the corresponding selection buffer only supplemented with blocking solution to act as a negative control. Incubate the mixture for 15 min on a vibrating platform (700 r.p.m.) at RT. Wash five times with 250 µl of the appropriate selection buffer.

57| For each selection condition, dispense 100 µl of the preincubated phage (from Step 54) into both a selection well and a control well, and incubate the plate for 2 h on a vibrating platform (700 r.p.m.). Carefully remove nonbound phage particles and wash each well 15 times by adding 250 µl of the selection buffer. After each wash step, remove excess liquid by tapping the emptied plates once on a paper towel, avoiding cross-contamination of the phage to the neighboring wells. After the final wash step, carefully remove excess liquid via pipetting.

58| To elute phage bound to the wells, add 100 µl of 0.25 mg ml⁻¹ trypsin solution to each selection well and the corresponding control well and incubate for 30 min at RT on a vibrating platform (700 r.p.m.). This step will dissociate the phage bound to the immobilized antigen by proteolysis without affecting phage infectivity. Transfer the eluted phage from each selection well and the corresponding control well to separate microcentrifuge tubes prefilled with 5 µl of a 4 mg ml⁻¹ AEBSF solution to inhibit protease activity.

▲ CRITICAL STEP Although one round of selection is often sufficient to identify binders, nonspecific binding limits the target-specific phage enrichment that can be achieved per selection round. Therefore, consecutive rounds of selection may be needed to select the binders with the desired properties from the immune library. Consider adapting the panning conditions to select Nanobodies with higher affinity or to bias the selection to an epitope of interest. Take into account that subsequent rounds of selection will reduce diversity.

59| To allow recovery of the phage that eluted from each well, infect 3 ml of exponentially grown TG1 cells (from Step 52) in a 50-ml Falcon tube with 50 µl of the eluted phage from Step 58 and incubate for 30 min at 37 °C without shaking. Add 7 ml of LB and supplement it with 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose; grow overnight at 37 °C and 170 r.p.m. Store 1-ml aliquots at −80 °C as a glycerol stock (20% (vol/vol)) for later use. Glycerol stocks of these selected sublibraries can be stored for multiple years without detectable loss of Nanobody repertoire diversity.

■ PAUSE POINT The phage can be rescued from this sublibrary at any time (Step 61).

60| To estimate the number of infective recombinant phage that eluted from each well, titrate the remaining eluted phage (from Step 58) according to Steps 47 and 50. For a first selection round, typically phage is diluted from 1 up to 10⁴-fold. If the amount of phage that eluted from the selection well is at least 100-fold higher than that eluted from an appropriate negative control well, we advise screening individual clones of this selection round for the desired properties (Step 62). When the enrichment factor is below 100, we recommend rescuing this sublibrary and proceeding with another selection round (Step 61).

▲ CRITICAL STEP Although one round of selection is often sufficient to identify binders, nonspecific binding limits the target-specific phage enrichment that can be achieved per selection round. Therefore, consecutive rounds of selection may be needed to select the binders with the desired properties from the immune library. Consider adapting the panning conditions to select Nanobodies with higher affinity or to bias the selection to an epitope of interest. Take into account that subsequent rounds of selection will reduce diversity.

61| Day 3: rescue of the eluted phage for consecutive rounds of panning. To rescue and amplify phage for a consecutive round of panning from the overnight-grown infected TG1 culture or the frozen glycerol stock (from Step 59), inoculate 500 µl into a baffled 250-ml Erlenmeyer flask containing 50 ml of 2× TY supplemented with 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose; grow the cells at 37 °C and 200 r.p.m. until they reach exponential phase. Repeat Steps 38–60.

Screening for antigen binders ● TIMING 4 d

62| To isolate individual clones from an enriched sublibrary, prepare tenfold serial dilutions of the overnight-grown phage-infected TG1 cells (Step 59) in sterile LB. Homogeneously spread 100 µl of the serial dilutions over individual culture plates containing selective medium (LB agar + 100 µg ml⁻¹ ampicillin + 2% (wt/vol) glucose) by using sterile glass beads and incubate the plates overnight at 37 °C. A volume of 100 µl of a 10⁵ dilution typically results in ~100 single colonies per plate.
63| We typically screen 96–192 individual clones per target in total and pick at least 24 candidates per enriched selection condition. To prepare master plates, inoculate single colonies into wells of a 96-well round-bottom culture plate filled with 100 µl of 2× TY supplemented with 100 µg ml⁻¹ ampicillin, 2% (wt/vol) glucose and 10% (vol/vol) glycerol per well. Inoculate one well with a TG1 transformant expressing an irrelevant Nanobody as the negative control, and assign one well without inoculum to monitor possible well-to-well contaminations. Grow overnight at 37 °C without shaking.  

**PAUSE POINT** We store these master plates containing glycerol stocks of *E. coli*–expressing monoclonal Nanobodies at −80 °C. Individual clones can be recovered from master plates that have been stored for years.

64| To prepare *E. coli* periplasmic extracts containing Nanobodies, inoculate 10 µl of an overnight-grown master plate in a 96-deepwell plate containing 1 ml of 2× TY + 100 µg ml⁻¹ ampicillin + 0.1% (wt/vol) glucose per well. Cover the plates with a sterile gas-permeable adhesive seal and incubate them for 3 h at 37 °C and 200 r.p.m. until the cells are in the exponential phase. Induce Nanobody expression by adding 100 µl of 10 mM IPTG in 2× TY per well, and shake for 4–6 h at 37 °C and 200 r.p.m. Pellet the cells at 3,200 g for 10 min. Discard the supernatant in a single movement by turning the plate upside down, followed by a simple sudden vertical move downward. Place the plate upside-down on a paper towel and freeze the cell pellets at −20 °C for 30 min.  

**PAUSE POINT** Frozen cell pellets can be stored at −20 °C for months without loss of Nanobody activity.

65| Thaw the deep-well plate containing cell pellets for 15 min at RT. To release the Nanobodies from the periplasm, add 100 µl of PBS to each well. Incubate the plate for 30 min at RT on a vibrating platform (700 r.p.m.). Pellet the cell debris by centrifuging for 10 min at 3,200 g and 4 °C. Carefully recover 90 µl of the supernatant without disturbing the cell pellet and transfer it to a new 96-well conical-bottom plate. The recovered solution typically contains up to 500 nM Nanobody.

66| As a first step, we screen for target specificity via ELISA according to the protocol described in Box 1. We routinely screen fivefold-diluted periplasmic extracts. Detect Nanobody binding by incubating with a His-tag–specific secondary antibody and an anti-mouse-AP conjugate according to the manufacturer's instructions. Use the DNPP reagent to develop the ELISA.  

**CAUTION** If the target also contains a His-tag, use an appropriate secondary antibody and conjugate.

7 TROUBLESHOOTING

67| Optionally, the same periplasmic extracts can be used in other biochemical, cellular or biophysical screens to further characterize the functional properties of the Nanobodies. For membrane-anchored targets, we suggest to also screen via flow cytometry to assess Nanobody specificity to the target in its native membrane environment.  

**Sequence determination ● TIMING 2–3 d**

68| Thaw the master plate (Step 63) and resuspend 5 µl of the cells expressing Nanobody with the desired properties in 45 µl of sterile water. Use 5 µl of this cell suspension as a template with vector-specific primers MP57 and GIII in the following PCR:

<table>
<thead>
<tr>
<th>Volume (µl)</th>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Template (cell suspension)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>10× KAPA Taq buffer A</td>
<td>1×</td>
</tr>
<tr>
<td>0.5</td>
<td>Forward primer MP57 (10 µM stock)</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>0.5</td>
<td>Reverse primer GIII (10 µM stock)</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>0.5</td>
<td>dNTPs (10 mM stock each)</td>
<td>200 µM</td>
</tr>
<tr>
<td>0.2</td>
<td>KAPA Taq DNA polymerase (5 U µl⁻¹ stock)</td>
<td>0.02 U µl⁻¹</td>
</tr>
<tr>
<td>15.8</td>
<td>ddH₂O</td>
<td></td>
</tr>
</tbody>
</table>

Amplify the DNA with the following PCR conditions:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>2 min</td>
</tr>
<tr>
<td>2–28</td>
<td>94</td>
<td>30 s</td>
</tr>
<tr>
<td>28</td>
<td>55</td>
<td>30 s</td>
</tr>
<tr>
<td>29</td>
<td>72</td>
<td>40 s</td>
</tr>
<tr>
<td>29</td>
<td>72</td>
<td>7 min</td>
</tr>
</tbody>
</table>

© 2014 Nature America, Inc. All rights reserved.
Analyze the PCR products via gel electrophoresis on a 1% (wt/vol) agarose gel. Clones expressing a genuine Nanobody will generate a fragment of ~700 bp. Determine the DNA sequence by using MP57 or GIII as sequencing primers. Group Nanobodies with a high similarity in their CDR3 sequence (identical length and >80% sequence identity) into sequence families. Nanobodies from the same family derive from the same B-cell lineage and bind to the same epitope on the target.

**Expression and purification of Nanobodies ★ TIMING 5 d**

1. **CAUTION** To obtain the highest yields, we advise expressing soluble Nanobody in the original display vector in the periplasm of *E. coli* strain WK6.

2. Inoculate a single colony derived from individual wells of the master plate (Step 63) in 5 ml of LB supplemented with 100 µg ml⁻¹ ampicillin and 2% (wt/vol) glucose; grow overnight at 37 °C. Prepare the plasmid from the TG1 cells by using the PureYield plasmid miniprep system. Transform the purified plasmid into *E. coli* WK6. Inoculate a single colony into a 50-ml sterile Falcon tube containing 10 ml of LB supplemented with 100 µg ml⁻¹ ampicillin, 2% (wt/vol) glucose and 1 mM MgCl₂. Grow the preculture overnight at 37 °C and 170 r.p.m.

3. Inoculate 330 ml of TB supplemented with 100 µg ml⁻¹ ampicillin, 0.1% (wt/vol) glucose and 1 mM MgCl₂ in a 1-liter baffled flask with 3 ml of the preculture. Shake the mixture at 37 °C and 170 r.p.m. until it reaches an OD₆₀₀ of 0.7. Induce Nanobody expression with 1 mM IPTG (final concentration) and grow overnight at 170 r.p.m. and 28 °C. Alternatively, induce Nanobody expression for 4 h at 170 r.p.m. and 37 °C.

4. Collect the bacteria by centrifugation for 15 min at 9,000g at RT. Carefully resuspend the cell pellet of 1 liter of culture in 15 ml of ice-cold TES, and incubate it for at least 1 h on ice on an orbital shaking platform. Add 30 ml of TES/4 buffer to the resuspended pellet, and shake it for 45 min on ice on an orbital shaking platform. Centrifuge the suspension for 30 min at 10,000g at 4 °C and recover the supernatant as the periplasmic extract.

5. Purify the His-tagged Nanobodies from the periplasmic extract by using IMAC according to the manufacturer’s instructions. This expression and purification protocol routinely yields 1–10 mg per liter of culture with an estimated purity of ≥95%. For crystallography-grade Nanobodies, a subsequent polishing step via size-exclusion chromatography is often required.

**TROUBLESHOOTING**

**TROUBLESHOOTING**

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

**Table 3** | Troubleshooting table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reasons</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>No serum conversion</td>
<td>Biotinylation denaturates target</td>
<td>Use a lower molar excess of biotin reagent for target biotinylation and assess folding (Step 55) Assess serum conversion with nonbiotinylated target</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low target immunogenicity</td>
<td>Immunize other animals and use an alternative immunogen format (<strong>Table 2</strong>). Because camelds are outbred, the magnitude of the serum conversion between animals may differ</td>
</tr>
<tr>
<td>22</td>
<td>No Nanobody repertoire amplified</td>
<td>RNA degraded</td>
<td>Collect new blood sample and extract PBLs (Steps 7–10), make new buffers, prepare fresh RNA (Steps 13–18) and verify the integrity of RNA via agarose gel electrophoresis</td>
</tr>
<tr>
<td>30</td>
<td>Library size &lt;10⁷ or insert percentage &lt;75%</td>
<td>Inefficient digestion by PstI-Eco91I</td>
<td>Evaluate integrity and concentration of digested vector and Nanobody repertoire before ligation via gel electrophoresis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low transfection efficiency</td>
<td>Assess transformation efficiency of TG1 with supercoiled plasmid. Optimize the vector to insert ratio Use fresh T4 DNA ligase</td>
</tr>
</tbody>
</table>

(continued)
**TABLE 3 | Troubleshooting table (continued).**

<table>
<thead>
<tr>
<th>Step</th>
<th>Problem</th>
<th>Possible reasons</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>Phage titer below $10^{12}$ c.f.u. per ml</td>
<td>Repression of lacZ promoter</td>
<td>Do not add glucose during overnight phage amplification (Step 39)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Low helper phage infectivity</td>
<td>Prepare new helper phage and measure infectivity (Step 38)</td>
</tr>
<tr>
<td>60</td>
<td>Enrichment &lt;10 after two panning rounds</td>
<td>Aspecific binding of phage</td>
<td>Use alternative blocking agent (e.g., BSA, Steps 53–56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein prep not homogeneous</td>
<td>Use a better purified protein sample for panning (Step 55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Biotinylation denatures target protein</td>
<td>Biotinylate target with lower molar excess of biotin reagent and assess folding (Step 55)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Repeat panning using an alternative target presentation method (Table 1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immune titer mediated by conventional IgGs</td>
<td>The presence of target specific Nanobodies in the each phage (sub)library can be addressed by performing an ELISA (Box 1) using phage from Step 45 as the sample in step 3 of Box 1. Use an M13 specific antibody conjugate for detection</td>
</tr>
<tr>
<td>66</td>
<td>No target-specific Nanobodies identified</td>
<td>Low Nanobody expression in TG1 periplasm</td>
<td>Perform western blotting to confirm that Nanobodies are expressed in Step 65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quality of the immobilized target</td>
<td>Confirm the quality of the immobilized target with an existing (conformational) antibody. Repeat ELISA using an alternative target presentation method (Table 1)</td>
</tr>
<tr>
<td>73</td>
<td>Poor Nanobody yield</td>
<td>Low expression</td>
<td>Use the WK6 strain for expression. Some Nanobodies have the intrinsic property to express poorly in <em>E. coli</em>. In this case, we recommend trying to express another Nanobody from the same family</td>
</tr>
</tbody>
</table>

**● TIMING**

Steps 1–3, protein storage and analysis: 1 d  
Steps 4–6, immunization: 6 weeks  
Steps 7–10, blood sampling and lymphocyte preparation: 1 d  
Steps 11 and 12, analysis of serum conversion: 2 d  
Steps 13–18, isolation of total RNA: 1 d  
Steps 19 and 20, cDNA synthesis: 4 h  
Steps 21–36, construction of the immune library: 5 d  
Steps 37–45, rescue and amplification of phage from immune libraries: 2 d  
Steps 46–61, antigen presentation and selection by panning: 3 d per round of selection  
Steps 62–67, screening for antigen binders: 4 d  
Steps 68 and 69, sequence determination of positive clones: 2–3 d  
Steps 70–73, expression and purification of Nanobodies: 5 d  
**Box 1, ELISA**: 2 d

**ANTICIPATED RESULTS**

Three representative examples of Nanobody discovery programs with distinct levels of complexity are presented below. Each program led to the identification of conformational Nanobodies that were instrumental to growing diffracting crystals that allowed high-resolution structures to be determined.

**Nanobody-assisted X-ray crystallography of BACE2**

Inhibition of the aspartic protease beta-site APP-cleaving enzyme 2 (BACE2) has recently been shown to lead to improved control of glucose homeostasis and to increased insulin levels in insulin-resistant mice. BACE2 may therefore be of high...
importance in drug discovery as a target for the expansion of functional pancreatic cell mass in diabetes. We generated a series of BACE2-specific Nanobodies for Nanobody-assisted crystallography to create a toolbox of well-diffracting BACE2 crystals grown from solutions with different pH and with different packing interactions and different active-site conformations to repeatedly and rapidly obtain cocrystal structures with previously intractable inhibitor series\(^2\).

A BACE2-specific humoral response was induced in two llamas. One llama was immunized with recombinant BACE2 and the other was injected with inhibitor-saturated BACE2 according to Steps 4–6. Both llamas were simultaneously immunized with two other target proteins. As the protein storage buffers of the three immunogens were not compatible, all targets were injected at separate locations. A display library was constructed in pMESy4 by using pooled RNA extracted from blood samples collected 4 and 8 d after the final antigen boost according to Steps 7–36.

Panning was performed on NeutrAvidin-captured biotinylated BACE2 in the presence of excess inhibitor in appropriate selection buffer (20 mM Bis-Tris propane (pH 7.0), 0.15 M NaCl, 10% (vol/vol) glycerol, 0.3% (wt/vol) CHAPS). Biotinylated BACE2 was prepared by using EZ-Link NHS-chromogenic-biotin according to the instructions of the manufacturer. After two rounds of panning, 129 out of 138 Nanobodies scored positive in an ELISA using NeutrAvidin-captured biotinylated BACE2 in the presence of excess inhibitor. After sequence analysis, 30 unique BACE2-specific Nanobodies were identified that belonged to nine sequence families. Between 1 and 13 sequence variants were identified per family.

Six Nanobodies, all belonging to different sequence families with an affinity between 1 and 100 nM and covering three distinct epitopes (as determined by surface plasmon resonance) were selected for cocryrstallization. XA4813 yielded six different crystal forms diffracting up to 1.5-Å resolution for both wild-type and mutant BACE2 (ref. 27). The Nanobody cocrystals allowed the determination of high-resolution structures of BACE2 inhibitor complexes that could not be obtained with other BACE2 crystals. A high-resolution structure of BACE2 in complex with two Nanobodies (XA4813 and XA4815) was also obtained.

**Nanobodies stabilizing the active \(\beta_2\) adrenoreceptor conformational state**

Efforts to obtain an agonist-bound active-state GPCR structure have proven to be difficult owing to the inherent instability of this state in the absence of a G protein. We generated Nanobodies to the human \(\beta_2\) adrenoreceptor (\(\beta_2\AR\)), which exhibits G protein–like behavior, and we obtained an agonist-bound, active-state crystal structure of the receptor-Nanobody complex\(^1\). For this purpose, \(\beta_2\AR\) truncated after amino acid 365 (\(\beta_2\AR-365\)) having an amino terminal Flag epitope tag was expressed in Sf9 insect cells and purified by sequential M1 antibody– and alprenolol affinity chromatography as described\(^6\). To reconstitute the receptor in lipid vesicles, purified \(\beta_2\AR-365\) was immobilized on an anti-Flag M1 column and equilibrated with 10 column volumes of a mixture of 5 mg ml\(^{-1}\) 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 0.5 mg ml\(^{-1}\) lipid A in 1% (wt/vol) octylglucoside, 100 mM NaCl, 20 mM HEPES (pH 7.5), 2 mM CaCl\(_2\), and 1 \(\mu\)M superagonist BI-167107. The \(\beta_2\AR\) was then eluted in the same buffer without CaCl\(_2\) supplemented with EDTA and Flag peptide. Lipid vesicles were then dialyzed against PBS containing 1 \(\mu\)M agonist at 4 °C to remove detergent.

The concentration of the reconstituted protein was adjusted to 5 mg ml\(^{-1}\), aliquotted and stored at −80 °C.

A single llama received six weekly administrations of the reconstituted truncated \(\beta_2\AR\) according to Steps 4–6. Lymphocytes were isolated from 50 ml of blood of the immunized llama, and total RNA was prepared from these cells according to Steps 7–18. The coding sequences of the Nanobody repertoire were amplified by RT-PCR and cloned into the phage display vector pMES4 according to Steps 19–36. \(\beta_2\AR\)-specific phage was enriched by two

---

**Figure 3** | Nb80 is a structural mimic of GoS and stabilizes the active-state conformation of \(\beta_2\AR\). Nb80 (red) and GoS (green) bind the same intracellular cavity of the agonist-bound \(\beta_2\) adrenoreceptor in the \(\beta_2\AR-Nb80\) complex\(^1\) (Protein Data Bank (PDB) 3P0G; \(\beta_2\AR\) in orange, Nb80 in red) and the \(\beta_2\AR-Gs\) complex\(^2\) (PDB 3SN6; \(\beta_2\AR\) in gray, GoS in green, G\(\beta\)S in salmon and G\(\gamma\) in blue).
rounds of in vitro selection on 96-well Maxisorp plates coated with the lipid vesicle–reconstituted β2AR-365 receptor according to Steps 37–61. Antigen-bound phage was recovered from antigen-coated wells either by adding triethylamine (pH 11) and neutralizing with Tris-HCl (pH 7), or by adding freshly grown TG1 E. coli cells.

After two rounds of biopanning, 96 individual colonies were randomly picked and the Nanobodies were produced as a soluble His-tagged protein in the periplasm of the TG1 cells. Solid-phase ELISA identified 16 different conformational Nanobodies that recognize native agonist-bound β2AR-365, but not the heat-denatured receptor. Seven clones recognized agonist-bound (BI-167107) but not inverse agonist-bound receptor (ICI-118,551). Of these, Nanobody Nb80 was chosen for cocrystallization experiments because it showed G protein–like properties upon binding to both wild-type β2AR and β2AR-T4L. Crystals of BI-167107-bound β2AR-T4L in complex with Nb80 were obtained in lipid bicelles and lipidic cubic phase (LCP) at pH 8.0 in 39–44% (wt/vol) PEG400, 100 mM Tris, 4% (vol/vol) DMSO and 1% (wt/vol) 1,2,3-heptanetriol. Nb80 binds to the cytoplasmic cavity of the β2AR, with the third complementarity-determining region (CDR3) loop projecting into the core of the receptor (Fig. 3).

Nanobodies stabilizing the β2AR-Gs multiprotein complex

The β2 adrenoreceptor activation of Gs, the stimulatory G protein for adenyl cyclase, has long been a model system for GPCR signaling. We used Nanobody-assisted X-ray crystallography to solve the crystal structure of the active-state ternary complex composed of agonist-occupied monomeric β2AR and nucleotide-free Gs heterotrimer. Formation of a stable β2AR-Gs complex from the Gs heterotrimer (Gαs, Gβ and Gγ) and BI-167107-bound β2AR-365 in 0.02% (wt/vol) MNG-3 (ref. 68) was accomplished as described2. MNG-3 (also known as NG310, Anatrace) is a detergent that has high avidity for the β2AR and a very slow dissociation rate compared with other detergents such as dodecyl maltoside. This is important for maintaining the native conformation of the receptor after immunization. From negative-stain electron microscopy imaging34, we observed that the α-helical domain of Gαs was flexible, and therefore it was possibly responsible for poor crystal quality. Targeted stabilization of this domain was addressed by immunizing two llamas with 100 µg of the bis(sulfo succinimidyl)glutarate (BS2G, Pierce) cross-linked BI167107-β2AR-Gs ternary complex, followed by 3 biweekly shots of 50 µg. After completing the immunization, PBLS were isolated from the immunized animals to extract total RNA and prepare cDNA according to Steps 13–20.

For each llama, a separate phage display library was constructed in pMESy4 (Steps 21–36). Nanobodies specific for the multiprotein complex were enriched by two rounds of panning on (i) the BI167107-β2AR-Gs ternary complex embedded in Apo A-I biotinylated high-density lipoprotein particles (rHDL) or (ii) on the BS2G cross-linked BI167107-β2AR-Gs ternary complex. For the first panning strategy, biotinylated rHDL particles containing the BI167107-β2AR-Gs ternary complex were immobilized on a NeutrAvidin-coated Maxisorp plate at 0.5 µM per well in 20 mM HEPES (pH 8.0), 100 mM NaCl, 1 mM EDTA, 100 µM TCEP and 100 nM BI167107. For the second panning strategy, the BS2G-cross-linked BI167107-β2AR-Gs ternary complex was solid phase–coated on an ELISA plate at 1 µg per well. To elute complex-specific phage, the wells were treated with trypsin according to Step 58. After two rounds of selection, 24 colonies were randomly picked from each enriched sublibrary, and the Nanobodies were expressed in the periplasm of E. coli according to Steps 64 and 65.

Two immunizations and two separate selection strategies lead to the discovery of eight unique sequence families that are specific for the complex. Six of these families enriched both on biotinylated rHDL particles containing the BI167107-β2AR-Gs ternary complex and on the solid phase–coated BS2G-cross-linked BI167107-β2AR-Gs ternary complex. None of the Nanobodies bound to β2AR-365 receptor alone. To identify Nanobodies providing stabilization to the G protein subunits, the agonist-β2AR-Gs ternary complex was analyzed by natural size-exclusion

Figure 4 | Structure of the β2AR-Gs complex solved by Nanobody-enabled X-ray crystallography. Surface representation of the active-state ternary complex composed of agonist-occupied monomeric β2AR (gray) and nucleotide-free Gs heterotrimer. Nb35 (red, cartoon representation) binds at the interface of the Gαs (green) and Gβ (salmon) subunits. Gγ is represented in blue.
chromatography in the presence and absence of the nonhydrolyzable GTP analog GTP\(\gamma\)S. It was found that Nb35 (refs. 2) and Nb37 (refs. 34,38) protect the BI167107-\(\beta_2\)AR-Gs complex from dissociation by GTP\(\gamma\)S.

The T4L-\(\beta_2\)AR-Gs-Nb35 complex was used to obtain crystals in LCP (7.7MAG) that diffracted to 2.9 Å. In the crystal structure, Nb35 packs at the interface of the \(\beta\)G and G\(\alpha\) subunits, with CDR1 interacting primarily with G\(\beta\) and a long CDR3 loop interacting with both G\(\beta\) and G\(\alpha\) subunits (Fig. 4).

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

ACKNOWLEDGMENTS We thank members and associates of the Steyaert, Muyldermans, Hol and Kobilka laboratories, past and present, for their assort contributions over the years to this work. In particular, we acknowledge the contributions of N. Buys and Y.Z. Park. The Steyaert laboratory was supported by the Fonds Wetenschappelijk Onderzoek-Vlaanderen through research grants G011110N and G049512N, Innoviris Brussels through the Impulse Life Science program BRGD0132, the Belgian Federal Science Policy Office through IAP7-40 and by the BBO program IWT120026 from the Flemish Agency for Innovation by Science and Technology. B.K.K. received support from US National Institutes of Health (NIH) grants R01NS028471 and R01GM083118 and from the Maters Foundation. The research in the laboratory of W.G.J.H. was supported by the National Institute of Allergy and Infectious Diseases (NIAID) and the National Institute of General Medical Sciences (NIGMS) of the NIH under award numbers AI34501 and GM77048. S.T. received a doctoral fellowship from the Fonds Wetenschappelijk Onderzoek-Vlaanderen. S.G.F.R is supported by the Lundbeck Foundation.

AUTHOR CONTRIBUTIONS J.S. developed the concept of Nanobody-assisted crystallography in collaboration with W.G.J.H. and B.K.K.; E.P., T.L., S.T., A.W. and J.S. worked out the protocol. E.P., T.L., S.M., W.G.J.H., B.K.K. and J.S. contributed to the Introduction. E.P., T.L., S.G.F.R., A.R., B.K.K. and J.S. developed the experiments described in the Anticipated Results and all authors participated in discussions on technical and conceptual aspects of the protocol and the editing of the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

17. Læremans, T. et al. Genetic immunization for producing immunoglobulins against cell-associated antigens such as P2X7, CXCR7 or CXCR4. WO patent 2,010,070,145 (2010).