Bispecific single domain antibodies as highly standardized synthetic calibrators for immunodiagnosis

Paula Segovia[1], Pedro Quinteros[2], Sergi Morais[2], Cesar Echaides[3], Ángel Maquieira[2*], Gabriel Lassabe[1*], Gualberto Gonzalez-Sapienza[1*].

[1]Cátedra de Inmunología, Facultad de Química, DEPBIO, Instituto de Higiene, Montevideo, Uruguay. [2]Departamento de Química, Universidad Politécnica de Valencia, Valencia, Spain. [3]Parque Lecoq, IMM, Montevideo Uruguay

*Corresponding Authors: Gualberto González, ggonzal@fq.edu.uy; Gabriel Lassabe, glassabe@fq.edu.uy; Angel Maquieira amaquieira@qim.upv.es

ABSTRACT

Commonly, serological immunoassays and diagnostic kits include reference standard reagents (calibrators) that contain the specific antibodies to be measured, which are used for the quantification of the unknown antibody contained in the sample. However, in some cases, such as the diagnosis of allergies or autoimmune diseases, it is often difficult to have sufficient quantities of these reference standards, and there are also limitations to their lot-to-lot reproducibility and standardization over time. To overcome this difficulty, this work introduces the use of surrogate recombinant calibrators formulated on the basis of two single domain antibodies (nanobodies) combined through a short peptide linker to produce a recombinant bispecific construct. One of the nanobodies binds to the cognate antigen of the target antibody and the second is specific for the paratope of the secondary detecting antibody. The bispecific nanobody inherits the outstanding properties of stability and low cost production by bacterial fermentation of the parent nanobodies, and once calibrated against the biological reference standard, it can be reproduced indefinitely from its sequence in a highly standardized manner. As proof of concept, we present the generation and characterization of two bispecific calibrators with potential application to the diagnosis of allergy against the antibiotics aztreonam and amoxicillin.

INTRODUCTION

Standards are essential components of many immunoassays. They contain the same analyte that is being measured by the test in the intended matrix, and are used as a reference to quantitate the analyte in the unknown samples. In the case of many diagnostic tests, the standard is a biological preparation (e.g. serum) that is used as a reference for the quantification of the biomarker of interest. Due to their complexity, these preparations cannot be fully characterized by physicochemical methods and are commonly assigned a value of arbitrary units (AU), which are then used to express the test results. To ensure an adequate level of harmonization, organizations such as the World Health Organization sponsor international multi-center studies to validate the use of biological reference materials and assign them a value in international units (IU) [1]. For antibody detection tests, these standards are reference sera of human or animal origin, and diagnostic test developers use them as a calibration reference to transfer their value to a new serum masterlot, which then constitutes the standard reagent of the new test [2]. However, due to their biological origin, the reference sera are finite and their substitution can give rise to reproducibility problems due to batch-to-batch variations. Furthermore, in some diagnostic situations the reference serum does not exist or it is difficult to ensure sufficient supply for its use as assay reagent, particularly for isotypes other than IgG. That is the case of obtaining IgM reactive sera to an infectious disease in the context of a population with a high degree of vaccination, or the limited availability of IgE for rare allergies. In these situations, an interesting alternative to advance in the standardization of the test results is the use of well-defined and easily reproducible reagents as surrogate standards (calibrators).

In diagnostic assays to monitor levels of specific antibodies, the replacement of the seropositive reference serum or plasma requires the design of a reagent that mimics the antigen-recognition ability of the specific antibodies being measured, while providing binding sites for the secondary antibody used for detection. The strategy commonly used for this purpose has been to combine the human Fc region of interest with the antigen-binding site of heterologous antibodies obtained by hyperimmunization with the test antigen. Initially, this was achieved by chemical conjugation of whole antibodies [3], but later these calibrators were produced recombinantly by joining the human Fc region of interest with Fab or scFv fragments specific for the antigen of interest to produce chimeric antibodies[4-6]. The Fc region of interest can also be combined with Fc receptors (FcγR), to build (FcγR)$_2$Fc chimeras that can be loaded with hyperimmune polyclonal antibodies to the target antigen [7]. While providing a solution to the problem of standardization, these constructs need to be expressed in eukaryotic systems and are laborious and expensive to produce. In addition, the scFv components often present difficulties associated with their low stability and high propensity to aggregate [8], which may require additional stabilization steps through optimization of the linkers, framework region, introduction of inter-chain disulfide bonds, etc. [9,10].

In this work we introduce the use of single domain antibodies (nanobodies) as modular elements to produce highly standardized recombinant calibrators or positive controls for immunoassays. These single domain antibodies are derived from the variable domain (VHH) of the heavy chain only antibodies found in camelids, and have several advantages over conventional antibody fragments [11]. Due to their monodomain nature, the loss of activity

produced by heavy and light chain shuffling does not occur as in the case of conventional antibodies, so their specificity is preserved intact during the selection process by phage display, as well as during their recombinant expression [12]. Besides being generated in a simpler way, nanobodies have superior physicochemical properties than conventional antibody fragments in terms of solubility and stability, and are easily produced by fermentation in *E. coli* [13,14]. A salient property of nanobodies is their ability to work as building blocks that can be fused in tandem to form bispecific antibodies. In this way, recombinant calibrators can be generated by combining a nanobody against the antigen of interest with a nanobody against the antigen binding site of the secondary antibody. The production of this type of recombinant calibrators is facilitated by the fact that it is fairly easy to generate anti-idiotype nanobodies, because the paratope of the monoclonal antibody used for immunization turns out to be the most immunogenic region for the immunized animal [15,16].

As proof of concept in this study we developed a recombinant calibrator for an allergy test for the antibiotic aztreonam and amoxicillin as outlined in **figure 1**. The chosen model represents a typical example in which, as it occurs for other allergies against antibiotics, the absence of reference sera makes it difficult to harmonize the results obtained in different laboratories.

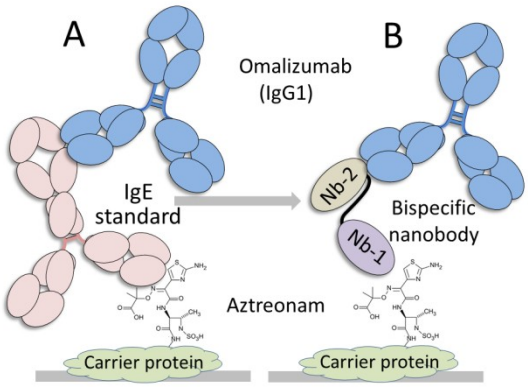


Figure 1. Schematic representation of the use of bispecific nanobodies as surrogate calibrators for biological standards. In the case of the immunodiagnosis of allergy to antibiotics, aztreonam in this example, the standard of IgE from patient sera is replaced by a bispecific nanobody composed of a nanobody that reacts with the adduct of the antibiotic, fused through a linker to a second nanobody that reacts with the paratope of the anti-IgE detection antibody (omalizumab).

MATERIAL AND METHODS

Materials. Bovine serum albumin (BSA), D-biotin, isopropyl β-D-1-thiogalactopyranoside (IPTG), LB Broth (Miller), 3,3´,5,5´ - tetramethylbenzidine (TMB), Tris base, Tween 20 and other chemicals were from Sigma (St. Louis, MO, USA). Anti-hemagglutinin epitope (anti-HA) antibody conjugated to horseradish peroxidase (HRP) was purchased from Sigma (Cat no. 12013819001), Anti-Histidine (anti-His) antibody conjugated to horseradish peroxidase (HRP) (Cat no. ab1187) and anti-human IgG antibody-HRP (Cat No. ab97225) were purchased from Abcam (Cambridge, UK), and streptavidin-HRP was purchased from Pierce (Rockford, IL, USA). Molecular biology reagents and *E. coli* One Shot™ BL21 (DE3) cells were from ThermoFisher Scientific (Waltham, MA, USA). *E. coli* ER2738 electrocompetent cells were purchased from Lucigen Corporation (Middleton, WI, USA). Omalizumab antibody was from Novartis International AG (Basel, Switzerland). HRP Conjugation Kit (Cat No. ab102890), from Abcam (Cambridge, UK) was used to prepare HRP-conjugated omalizumab. The stocks of each Ig class and subclass (Cat nos. 090701, 090704-M, 090704-1 M, 090705, 090707-2 M, 090707-4 M, 090713) were from Athens Research & Technology, Inc. (Athens, GA, USA). Chromatography columns were from GE Healthcare (Piscataway, NJ, USA). For the design of genes and primers, SnapGene software was used (from Insightful Science; available at snapgene.com). Primers and genes were obtained from General Biosystems Inc. (Morrisvile, NC, USA). The β-lactam antibiotics were conjugated to keyhole lympet hemocyanin (KLH) or human serum albumin (HSA) as described by Quintero-Campos *et al*. [17].

Library construction. A 3-year-old llama (*Lama glama*) from Lecocq Municipal Park Zoo (Montevideo) was immunized with 4 doses of 900 μg of purified omalizumab and 500 μg of Aztreonam and Amoxicillin antibiotics conjugated to HSA protein (human serum albumin) in incomplete Freund adjuvant by sub-cutaneous injection. Ten days after the third booster, 200 mL of blood was drawn. Peripheral blood mononuclear cells were isolated by Histopaque-1077 gradients (Sigma, St. Louis, MO, USA) according to manufacturer´s instructions, total RNA from 10^8 cells was extracted using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), quantified spectrophotometrically and reverse transcribed using oligo dT and RevertAID Reverse Trasncriptase (ThermoFisher Scientific, Waltham, MA, USA). Then the genes of the heavy chain variable domains of the conventional (VH) and heavy chain only antibody (VHH) isotypes were amplified by PCR as previously described [18]. The VH/VHH gene products were SfiI-digested, cloned in phagemid vector pComb3X (from Dr. Barbas, The Scripps Research Institute, La Jolla, USA) and electroporated into competent *E. coli* ER2738 cells. The transformed cells were cultured and superinfected with helper phage M13KO7 (New England Biolabs, Ipswich, MA, USA) at the beginning of the exponential phase to generate the phage library [18].

Panning for the selection of anti-omalizumab and anti-antibiotic nanobodies. The panning for selection of anti-omalizumab nanobodies involved a first step of pre-adsorption to remove reactive clones against epitopes other than the paratope of omalizumab. Pre-adsorption was performed by incubating 1×10^{11} colony-forming units (cfu) of the VH/VHH library on high-binding 8-well strips (Greiner Bio- One, Monroe, NC) coated with 1 μg/well of human IgG1, for 1 h at room temperature (RT). Phage supernatants were recovered and added to 4 wells coated with 1 μg/well of omalizumab and incubated for 2 h at room temperature. Wells were washed 10 x with PBS-0.05% Tween 20, followed by an incubation of 30 min at room temperature with PBS-0.05% Tween 20 and washed a further 10 x. The elution of bound phages was carried out by adding 50 μL/well of 10 mg/mL trypsin and incubating for 30 min at 37 °C with agitation. Finally, the phage output was titrated and used for subsequent amplification in *E. coli* ER2738 for a second round of selection.

Panning for selection of nanobodies against β-lactam antibiotic determinants didn´t require a pre-adsorption step and was carried out as described above, in well strips coated with 0.5 μg/well antibiotic conjugated to KLH protein.

High-throughput expression of nanobodies. DNA from the final/second output of the panning of omalizumab was amplified by infecting 0.5 mL of cell culture (OD600nm = 1) with 50 μL of output phage. Infected cells were diluted in a flask containing 9.5 mL of SB broth with 20 μg/mL ampicillin and cultured overnight at 37 °C. Plasmid DNA was purified using GeneJet Miniprep Kit (ThermoFisher Scientific, Waltham, MA, USA), SfiI-digested and cloned into the pINQ-BtH6 vector . The ligation product was electroporated into competent *E. coli* BL21(DE3) cells carrying the pCY216 vector for overexpression of the biotin ligase of *E. coli* and parallel cultures of individual colonies were then produced as described before [19]. Briefly, 89 colonies were inoculated to 500 μL of LB medium containing 50 μg/mL kanamycin, 35 μg/mL chloramphenicol, 0.04% of L-arabinose, and 100 μM D-biotin in 96-deep-well culture blocks (Greiner Bio-One, Monroe, NC). The expression of biotinylated nanobodies was induced at OD600nm = 0.6 with 10 μM IPTG overnight at 28 °C with shaking. The bacterial pellets were harvested by centrifugation, resuspended in PBS, lysated by four freeze-thaw cycles followed by 30 min of sonication in sonicator bath, and incubated for 2 h at 37 °C to promote efficient biotinylation. Cell lysates were centrifuged and the supernatants were collected to test their reactivity in ELISA. Each supernatant was analyzed in ELISA plates coated with 0.2 μg/well of omalizumab, human IgG1 and human IgG, and bound nanobodies were then detected with a streptavidin-HRP conjugate (Pierce, Rockford, IL).
The screenings of anti-antibiotic determinant nanobodies were performed by directly picking isolated colonies from the second round of panning. In this case, individual colonies were cultured/grown in SB broth-ampicillin and nanobody expression was induced with 1 mM of IPTG overnight at 37°C. After centrifugation the supernatants were collected and assayed in ELISA plates coated with 0.2 μg/well KLH-conjugated antibiotic aztreonam, meropenem, ceftriaxone, amoxicillin or penicillin. Bound nanobodies were then detected with an anti-HA-HRP conjugate.

Construction of the pINQ-bis vector. The pINQ-bis is an in house modified version of the pET-28a(+) vector that allows the expression of bispecific nanobodies in the periplasm of *E. coli* BL21(DE3). The modification resulted in an expression cassette coding for ompA signal peptide, the anti-β-lactam Nb flanked by two SfiI restriction sites, a spacer of GQAGR$(GGGGS)_3$TSEL, the anti-omalizumab Nb and a 6xHis tag. To exchange between anti-β-lactam nanobodies, the coding sequence of each nanobody was amplified by PCR using FwSfiI (5´-3´ seq: GGCCCAGGCGGCCATGG) and RvSfiI (5´-3´ seq: GGCCGGCCTGGCCTGAGG) and the obtained product was SfiI-digested and cloned into the pINQ-bis vector.

Expression and purification of the aztreonam/omalizumab (Az/Om) calibrator. *E. coli* BL21(DE3) cells were transformed with the pINQ-bis vector and seeded in LB agar-kanamycin plates. Two hundred mL of LB-kanamycin were inoculated with 2 mL of overnight culture of a single colony, and grown at 250 rpm, 37 °C. When OD600nm = 0.6, IPTG was added to a final concentration of 10 μM and cells were grown overnight at 28 °C. The following day, periplasmic extracts were prepared as described by Olichon et al. [20], supplemented with NaCl and imidazole up to 0.3 M and 20 mM, respectively, and the bispecific Nb was purified on Ni-NTA columns using AKTA purification system (GE Healthcare, Uppsala, Sweden). The eluted fractions were dialyzed against PBS, concentrated up to 250 μL using concentrator 9K MWCO (89884A, ThermoFisher Scientific, Waltham, MA, USA) and purified by size exclusion chromatography in PBS-0.2% Tween. This second purification was carried out using a Superose 12 HR 10/30 column (Cat no. 17-0538-01, Amersham, Uppsala, Sweden) in the AKTA

purification system. The MW standard proteins Ferritin (440 kDa), Aldolase (160 kDa), Ovoalbumin (45 kDa), α-Chymotrypsinogen A (25 kDa) and Ribonuclease (14 kDa) were used to build an elution-time calibration curve using the UV detector.

Binding analysis of nanobody interactions by bio-layer interferometry. Binding interactions were analyzed using the Blitz system (Fortebio, Inc., Menlo Park, CA). Kinetic rate constants were determined with Amine reactive biosensors (AR2G) according to manufacturer´s instructions. Omalizumab was immobilized on the sensors at 30 µg/mL through *N*-hydroxysuccinimide/1-ethyl-3-(3dimethylaminopropyl) carbodiimide (NHS/EDC) linkage and then the free amine reactive sites were blocked with 1 M ethanolamine, pH 8.5. The biosensors were then exposed for 120 s to five different concentrations of Nb E5 and Bispecific Nb Az/Om (25 nM, 50 nM, 100 nM, 200 nM, 400 nM) diluted in kinetic buffer (PBS with 0.2% Tween20, 1% BSA and 0.05% sodium azide), followed by a 120 s dissociation step in kinetic buffer while shaking at 2200 rpm. Interferometry data were globally fitted to a 1:1 binding ratio for calculating kinetic parameters using the BlitzPro Software, version 1.2 (ForteBio Inc., USA).

ELISA immunoassays to study the reactivity of monospecific and bispecific nanobodies. ELISAs were performed in 96-well microtiter high-binding plates (Cat No. 655061, Greiner Bio-One, Frickenhausen, Germany). All HRP conjugated reagents (referred in materials) were prepared according to manufacturer's instructions. Samples and reagents were incubated in 100 µL/well of PBS-0.1% BSA (dilution buffer) for one hour at RT with agitation. Peroxidase activity was measured using a substrate solution of TMB and H_2O_2 and absorbance was read at 450 nm with Fluostar Optima Reader (BMG, Ortenberg, Germany).
The anti-antigen reactivity of the purified monospecific and bispecific nanobodies was evaluated in ELISA plates coated with 0.3 µg/well of omalizumab or KLH-conjugated antibiotic. Antigen-bound monospecific nanobodies were detected by incubating wells with anti-HA antibody-HRP or streptavidin-HRP, whereas bispecific nanobodies were detected with anti-His antibody-HRP or by successive steps of 10 µg/mL omalizumab followed by anti-IgG antibody-HRP. For inhibition ELISAs, performed to evaluate specificity of nanobodies against the paratope of omalizumab, serial dilutions of nanobodies were co-incubated with 1.4 ng/mL of omalizumab in plates coated with 0.1 µg/well of IgE. Bound omalizumab was then detected with anti-IgG antibody-HRP.
The titration curve of the Az/Om calibrator was compared to that performed with an Artificial human serum (cIgE) (Dr. Fooke Laboratorien, Neuss, Germany), which is obtained by combining a fusion of the Fc domain of IgE and FcγRI (CD64) with aztreonam-specific polyclonal rabbit IgG, and has an equivalence of 8.5 IU/mL of aztreonam-specific IgE [7].

Analysis of human serum samples containing amoxicillin-specific IgE using amoxicillin/omalizumab (Amx/Om) calibrator. The Amx/Om was used as a calibrator in CLIA luminescence immunoassay for the determination of amoxicillin-specific IgE levels in serum samples. The assay was performed as described by Quintero-Campos et al. [17]. Briefly, white flat-bottomed polystyrene ELISA plates were coated with 0.3 µg/well of KLH-amoxicillin, incubated with 25 µL/well of serum samples or Amx/Om calibrator for 30 min at RT, and subsequently incubated with 25 µL/well of Omalizumab-HRP solution. Peroxidase activity was measured with chemiluminescent SuperSignal ™ ELISA Femto Substrate (ThermoFisher Scientific, Waltham, MA, USA), and luminescence signal was read at 450 nm using a multimode plate reader . A calibration curve was built by serial dilutions of the Amx/Om calibrator in IgE free serum (Cat no. H4522, Sigma, St. Louis, MO, USA) and it was used to quantitate amoxicillin-specific IgE of seven de-identified serum samples from allergic patients. These

samples were also analyzed with the ImmunoCAP specific IgE assay (ThermoFisher Scientific, Waltham, MA, USA) as a reference method.

RESULTS AND DISCUSSION

Selection of the anti-omalizumab nanobody. For the generation of the nanobodies (Nbs) to be used for the construction of the recombinant calibrator, a llama was immunized with the omalizumab antibody and the antigenic determinants of aztreonam and amoxicillin corresponding to the adducts formed through the reaction of their lactam ring with human albumin. After the fourth immunization, a VH/VHH library of 10^8 transformants was generated from 10^7 blood mononuclear cells. For the selection of the anti-omalizumab nanobody, in each round of panning the library was initially incubated in wells of a microtiter plate coated with human IgG1 (the isotype of the omalizumab antibody) in order to remove the nanobodies that could have been generated against epitopes that were not specific to the paratope of omalizumab. The VHH pool selected from the second round of panning was then cloned *en masse* into the SfiI sites of the pINQ-BtH6 plasmid for high yield expression and *in vivo* biotinylation. Initially, 89 clones were screened against omalizumab and selected on the basis of the strength of their reactivity at high dilution ($1/10^7$), which correlates with a relative high affinity/level of expression of the individual clones, **figure S1**.

Then, 34 of the most reactive clones were tested at a $1/10^2$ dilution against omalizumab, human IgG1 and total IgG. None of the clones showed cross-reactivity with these antigens indicating that preincubation of the library with human IgG1 efficiently removed clones that were not specific for the omalizumab paratope, **figure S2**. Sequencing of 10 of these clones revealed 5 unique sequences (**figure 2A**), all of them showed good level of expression (**figure 2B** and **2C**). Clones B6, E5 and E9 were expressed, purified by Ni-NTA agarose, and characterized for their reactivity against different classes of immunoglobulins. As shown in **figure 2D,** the three Nbs showed complete absence of reactivity against all immunoglobulin classes, adding support to the fact that they react against the paratope of omalizumab (**figure 2D**), as well as their potential to be used as a diagnostic reagent for clinical samples. The specificity of the three Nbs for the paratope of omalizumab was confirmed by inhibition tests, which also allowed us to rank the relative affinity of the Nbs, **figure 3**. Sub-nM concentrations of the Nbs were sufficient to inhibit 50% of maximum binding (IC_{50}); Nbs B6 and E5 have the highest relative affinity for omalizumab and the latter was chosen to construct the bispecific

calibrators due to its higher level of expression.

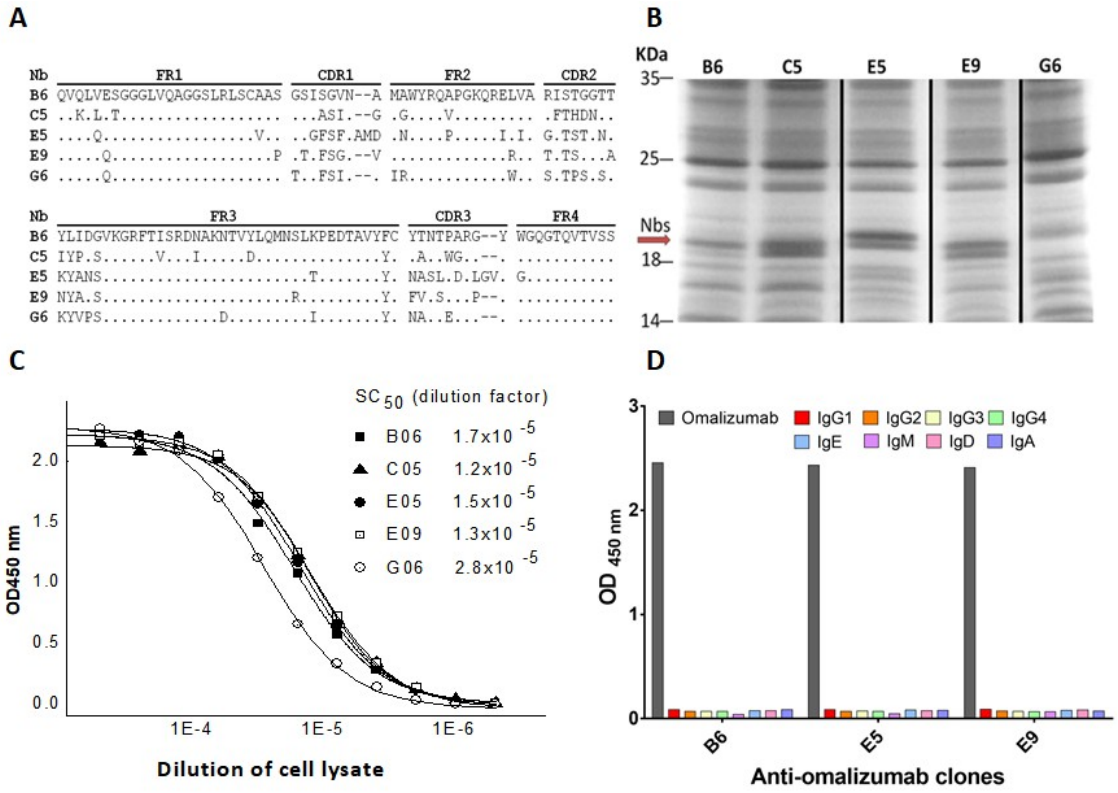


Figure 2. Sequences, expression and reactivity of selected Nb clones. A) Nb sequence alignment. Dots represent identity to the sequence of NbB06, gaps are represented by dashes. Framework (FR) and complementarity determining regions (CDR) are detailed at the top. B) SDS-PAGE corresponding to the expression of the Nbs in BL21 cells. C) Relative expression levels evaluated by reactivity in ELISA against omalizumab. D) Reactivity of the Nbs against different classes and subclasses of immunoglobulins was tested at saturation concentration.

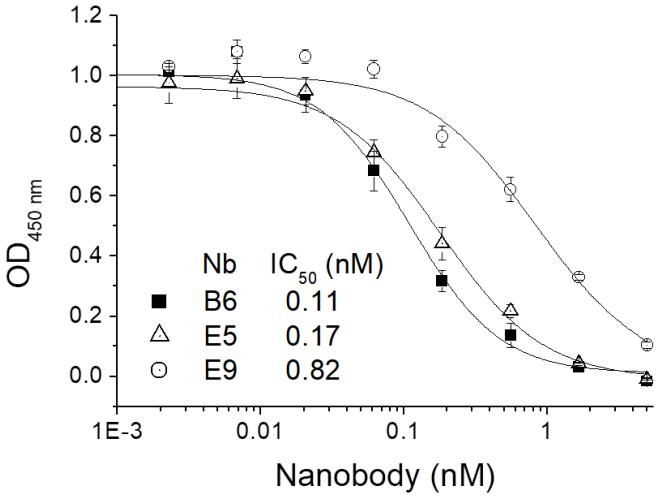


Figure 3. Inhibition curves of the interaction of omalizumab with IgE. The inhibition curves were made by co-incubating serial dilutions of the Nbs with fixed amounts of omalizumab in ELISA plates coated with human IgE, and then detecting the binding of omalizumab with an anti-human IgG-HRP conjugate. IC_{50} values are shown in the insert. Measurements were done in triplicates.

Selection of the anti-aztreonam nanobody

As a first example, a bispecific Nb was generated for the diagnosis of allergies to the antibiotic aztreonam. Selection for anti-aztreonam Nbs was performed from the nanobody library described above on microplates coated with the KLH-conjugated antibiotic. After two rounds of panning, ten clones were picked. In addition to testing the reactivity of these clones against aztreonam, the cross-reactivity with other β-lactam antibiotics was also evaluated, because a nanobody capable of reacting with different antibiotics could allow the same calibrator to function in more than one test, **figure 4**. In general, there was no cross-reactivity with the carrier protein or with the antigenic determinants of other antibiotics, except in the case of penicillin G, which despite a major structural difference with aztreonam presented some degree of cross-reactivity with many of the clones. However, due to its low intensity, this cross-reactivity with penicillin G would have no practical value, so the anti-aztreonam 01 Nb was finally selected for the construction of the bispecific antibody, based on its relative highest affinity/expression level, **figure S3.**

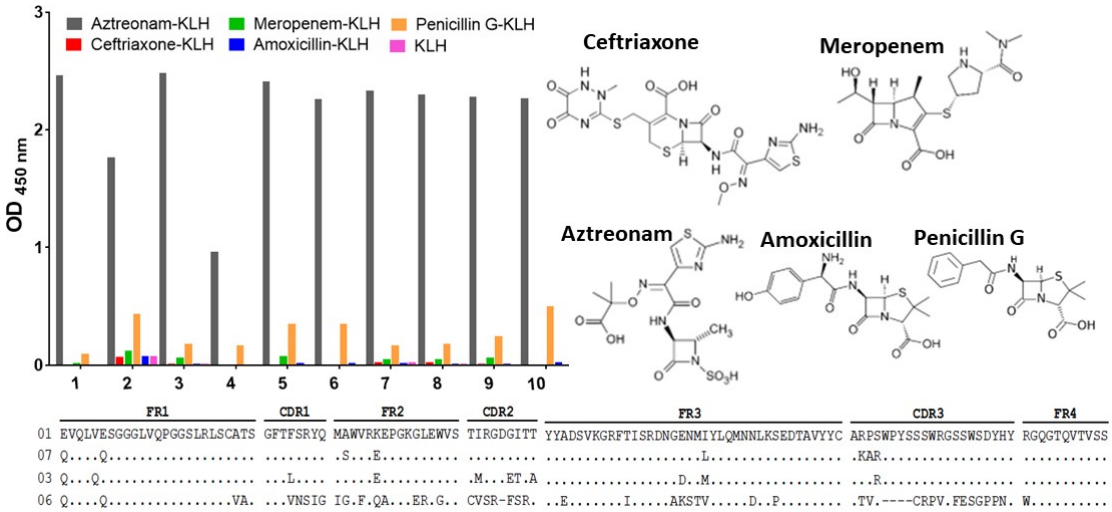


Figure 4. Selection of clones expressing anti-aztreonam nanobodies. The supernatants of 10 clones from the 2nd round of panning were tested by ELISA to analyze their reactivity against aztreonam-KLH and cross-reactivity against KLH and other β-lactam antibiotic determinants. The sequence alignment of four of the selected clones is shown below.

Construction of a synthetic calibrator for aztreonam

The scheme of the expression cassette used for the generation of the bispecific nanobody is shown in **figure 5**. The bispecific Nb was designed considering the possibility of an adaptable modular use for the diagnosis of allergies to β-lactam antibiotics, using the same anti-IgE detecting antibody (omalizumab in our case). For this purpose, the nanobody against the omalizumab paratope was fixed in the C-terminal region of the bispecific antibody, while in the N-terminal region a site was generated for the cloning of the Nb against the target antibiotic (anti-aztreonam in this example). This cloning site is flanked by the two SfiI sites present in the phage nanobody library, so that once the Nb has been selected against the β-lactam antibiotic of interest, it is easily inserted into the expression cassette of the bispecific calibrator.

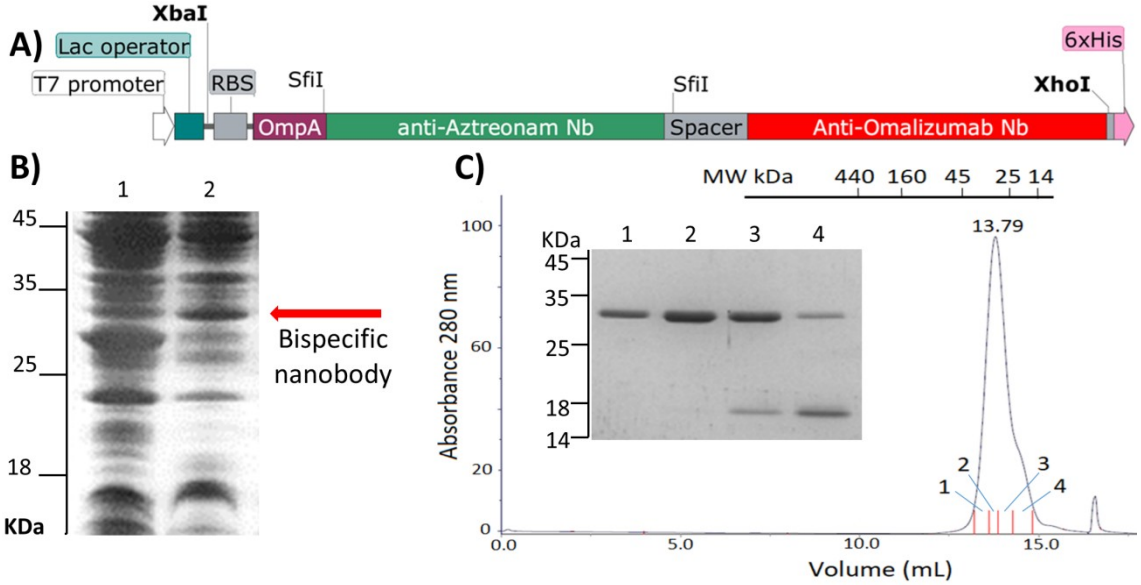


Figure 5. Expression and purification of the bispecific nanobody. A) Cloning cassette inserted between the XbaI and XhoI sites of the pET-28a(+) and used to express the bispecific calibrator (RBS, ribosome binding site; OmpA, signal peptide, Spacer: GQAGR$(GGGGS)_3$TSEL). B) SDS-PAGE analysis of transformed *E. coli* BL21 soluble cell extract before (1) and after IPTG induction (2). C) Gel filtration analysis of the Ni-NTA purified bispecific Nb; the elution volume of the main peak corresponds to the expected molecular size of the calibrator (about 32 kDa). The insert displays the SDS-PAGE analysis of the peak fractions.

The pET-28a(+) plasmid containing the cassette with the 01 Nb to aztreonam and the E5 Nb to omalizumab was transformed into BL21 *E. coli* cells to produce the aztreonam/omalizumab (Az/Om) calibrator. The recombinant calibrator was purified from the periplasmic extract and purified by affinity chromatography on Ni-NTA-agarose and gel filtration, **figure 6B-C**. The Az/Om calibrator was highly pure and did not associate in solution. There was no significant loss of reactivity of the individual Nbs in the bispecific construct as resulted from the comparison of the titration curves of the monomeric Nbs and the calibrator, **figure S4A and S4B**. Considering that the reactivity of the anti-omalizumab Nb could be affected by the interference of the spacer attached to its N-terminal residue, we further studied the preservation of its reactivity by biolayer interferometry (BLI) affinity measurements, **figure S4C and S4D**. There were no substantial changes in the affinity of monomeric (K_D = 7.19 nM) or

bispecific Nb (K_D = 6.97 nM), which is in agreement with the result of the titration curves, and indicates that the length and flexibility of the spacer do not compromise the recognition of the IgE epitope by the C-terminal Nb.

To simulate the shelf life of the calibrator in the final dilution of use in the assay, an accelerated stability study was carried out. The same calibrator preparation was stored at 4 and 37 °C for one and two weeks. As shown in **figure 6A**, there were no significant differences in the performance of the reagent stored under different conditions, confirming that, as expected, the bispecific construct is very stable, which is a very relevant property for its use as a diagnostic reagent. Due to the lack of international standards for aztreonam-specific IgE, the molar equivalence of the Az/Om calibrator in international units of IgE was established based on its correlation with the aztreonam surrogate standard, **figure S5** and **figure 6B**. On this basis, a 1.87 pM concentration of the Az/Om calibrator corresponds to 1 IU/mL of aztreonam-specific IgE. A more direct correlation could be obtained using sera from patients whose specific IgE has been quantified using a reference method as described below for the detection of amoxicillin.

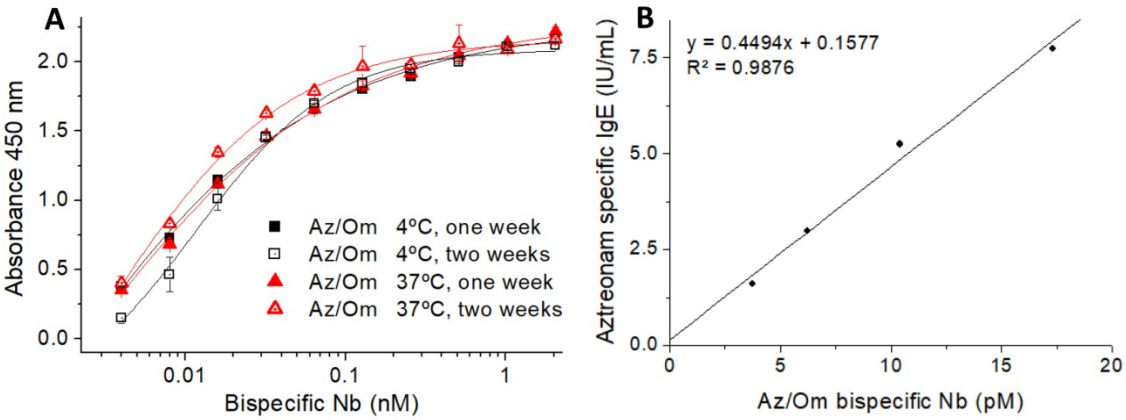


Figure 6. Stability and equivalence of the Az/Om calibrator. A) Accelerated stability of the Az/Om calibrator performed with the bispecific nanobody at the final concentration of use stored at different temperatures. Measurements were done in triplicates. B) Correlation curve of Az/Om concentration with the surrogate aztreonam-specific IgE standard.

Construction of a synthetic calibrator for amoxicillin

In a second application, a bispecific antibody was generated for the diagnosis of allergy to amoxicillin. Selection of the anti-amoxicillin Nb was performed in a similar way to that described for aztreonam using the amoxicillin-KLH conjugate. As shown in **figure 7A**, clones B56, B59 and B10 showed cross-reactivity with all β-lactams tested except for ceftriaxone. These clones could be used to prepare bispecific Nbs that could function as a common calibrator for these four antibiotics, however, we preferred to opt for a clone with higher specificity and chose clone 1, hereinafter called D51, due to its favorable expression. In a similar way to that described for the Az/Om calibrator, this Nb was used to produce the bispecific Nb D51/E5 (Amx/Om).

The bispecific Amx/Om Nb was diluted in IgE-free serum to build a calibration curve on ELISA plates coated with amoxicillin-KLH, using HRP-conjugated omalizumab and the SuperSignal ™ ELISA Femto Substrate (Thermo Fisher Scientific), **figure 7B**. In parallel, a panel of 7 samples of sera from patients with allergy to amoxicillin that had been previously analyzed using the ImmunoCAP® system (Thermo Fisher Scientific) was tested with this assay. **Figure 7C** shows that an excellent correlation was obtained between the IU/mL of amoxicillin-specific IgE measured by the reference technique and the corresponding concentration of the Amx/Om

calibrator obtained from the curve of **figure 7B**. On the basis of this correlation 259 pM of the Amx/Om calibrator corresponds to 1 IU/mL of amoxicillin-specific IgE.

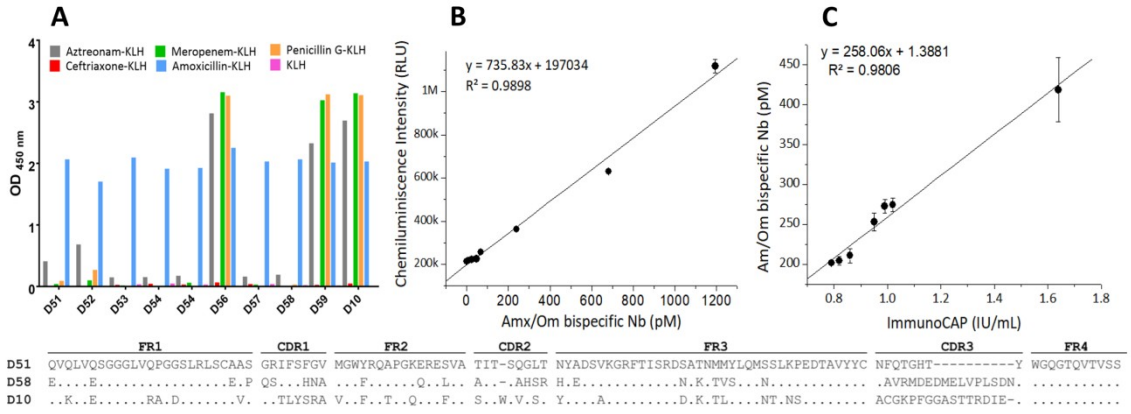


Figure 7. Nbs to amoxicillin and performance of the Amx/Om bispecific Nb. A) The supernatants of 10 clones from the 2nd round of panning were tested by ELISA to analyze their reactivity against amoxicillin-KLH and cross-reactivity against KLH and other β-lactam antibiotic determinants. The sequence alignment of three unique sequences is shown below. B) Chemiluminescent ELISA, calibration curve obtained with the Amx/Om bispecific Nb. C) Correlation between the amoxicillin-specific IgE concentration determined by the ImmunoCAP® system and the Amx/Om concentration.

CONCLUSIONS

The present study constitutes a proof of concept of the potential use of bispecific nanobody systems as substitutes for calibrators or positive controls based on biological materials. At present, the technology for generating nanobodies is highly developed and many laboratories have the capacity to produce them. A key component of the bispecific reagent is the Nb that reacts specifically with the paratope of the secondary antibody used for detection. In practice, due to the comparatively higher immunogenicity of the paratope, obtaining nanobodies with this specificity is undemanding, and can be further facilitated by including a pre-adsorption step on unrelated antibodies of the same isotype during panning. The other component is a second nanobody that reacts with the target antigen, which is even easier to obtain. Once calibrated against a reference standard or reference method, the bispecific calibrator has the potential to replace biological standards, without restrictions regarding its nature, whether it is proteins, lipids, polysaccharides, etc. In addition to their outstanding stability and low cost production by bacterial fermentation, nanobodies can be indefinitely reproduced in a homogeneous way from their sequence, offering a superb opportunity not only to improve the standardization of allergy tests, but also to overcome the difficulties of batch-to-batch reproducibility of many other diagnostic tests.

Finally, it is interesting to note that the use of bispecific Nbs as highly standardized reference standards as shown here can also be extended to other applications. Indeed, for example, in the case of two-site tests for the detection of biomarkers, if the biomarker standard is labile, unstable, difficult to produce or expensive, it could be replaced by a surrogate antigen consisting of a bispecific construct made up of two nanobodies against the individual paratope

of the pair of antibodies used in the test. Such a construct could also be useful as a standard in homologous or heterologous agglutination assays.

ACKNODLEDGMENTS

This work was supported by CSIC 2007-348, ANII FMV 2019_156321 and ANII FMV 2018_148245. PS is a recipient of a scholarship from ANII-Uruguay.

REFERENCES

(1) WHO Expert Committee on Biological Standardization. Sixty-fifth report. *World Health Organ Tech Rep Ser.* **2015**, 1-262.
(2) Broughton, P. M.; Eldjarn, L. Methods of assigning accurate values to reference serum. Part 1. The use of reference laboratories and consensus values, with an evaluation of a procedure for transferring values from one reference serum to another. *Ann Clin Biochem.* **1985**, *22 (Pt 6)*, 625-634.
(3) 1994.
(4) Hackett, J.; Hoff-Velk, J.; Golden, A.; Brashear, J.; Robinson, J.; Rapp, M.; Klass, M.; Ostrow, D. H.; Mandecki, W. Recombinant Mouse-Human Chimeric Antibodies as Calibrators in Immunoassays That Measure Antibodies toToxoplasma gondii. *Journal of Clinical Microbiology.* **1998**, *36*, 1277-1284.
(5) Hamilton, R. G. Engineered human antibodies as immunologic quality control reagents. *Annales de biologie clinique.* **1990**, *48*, 473-477.
(6) Tu, B.; Ziemann, R. N.; Tieman, B. C.; Hawksworth, D. J.; Tyner, J.; Scheffel, J.; Pinkus, M. S.; Brophy, S. E.; Werneke, J. M.; Gutierrez, R. Generation and characterization of chimeric antibodies against NS3, NS4, NS5, and core antigens of hepatitis C virus. *Clinical and Vaccine Immunology.* **2010**, *17*, 1040-1047.
(7) Offermann, N.; Plum, M.; Hubner, U.; Rathloff, K.; Braren, I.; Fooke, M.; Spillner, E. Human serum substitution by artificial sera of scalable allergen reactivity based on polyclonal antibodies and chimeras of human FcgammaRI and IgE domains. *Allergy.* **2016**, *71*, 1794-1799.
(8) Röthlisberger, D.; Honegger, A.; Plückthun, A. Domain interactions in the Fab fragment: a comparative evaluation of the single-chain Fv and Fab format engineered with variable domains of different stability. *J Mol Biol.* **2005**, *347*, 773-789.
(9) Jordan, J. L.; Arndt, J. W.; Hanf, K.; Li, G.; Hall, J.; Demarest, S.; Huang, F.; Wu, X.; Miller, B.; Glaser, S.; Fernandez, E. J.; Wang, D.; Lugovskoy, A. Structural understanding of stabilization patterns in engineered bispecific Ig-like antibody molecules. *Proteins.* **2009**, *77*, 832-841.
(10) Mabry, R.; Lewis, K. E.; Moore, M.; McKernan, P. A.; Bukowski, T. R.; Bontadelli, K.; Brender, T.; Okada, S.; Lum, K.; West, J.; Kuijper, J. L.; Ardourel, D.; Franke, S.; Lockwood, L.; Vu, T.; Frank, A.; Appleby, M. W.; Wolf, A.; Reardon, B.; Hamacher, N. B.; Stevens, B.; Lewis, P.; Lewis, K. B.; Gilbertson, D. G.; Lantry, M.; Julien, S. H.; Ostrander, C.; Chan, C.; Byrnes-Blake, K.; Brody, J.; Presnell, S.; Meengs, B.; Levin, S. D.; Snavely, M. Engineering of stable bispecific antibodies targeting IL-17A and IL-23. *Protein Eng Des Sel.* **2010**, *23*, 115-127.
(11) Muyldermans, S. Nanobodies: natural single-domain antibodies. *Annu Rev Biochem.* **2013**, *82*, 775-797.
(12) Gonzalez-Sapienza, G.; Rossotti, M. A.; Tabares-da Rosa, S. Single-Domain Antibodies As Versatile Affinity Reagents for Analytical and Diagnostic Applications. *Front Immunol.* **2017**, *8*, 977.
(13) Pérez, J. M.; Renisio, J. G.; Prompers, J. J.; van Platerink, C. J.; Cambillau, C.; Darbon, H.; Frenken, L. G. Thermal unfolding of a llama antibody fragment: a two-state reversible process. *Biochemistry.* **2001**, *40*, 74-83.

(14) Tabares-da Rosa, S.; Wogulis, L. A.; Wogulis, M. D.; Gonzalez-Sapienza, G.; Wilson, D. K. Structure and specificity of several triclocarban-binding single domain camelid antibody fragments. *J Mol Recognit.* **2019**, *32*, e2755.
(15) Alvarez-Rueda, N.; Ladjemi, M. Z.; Béhar, G.; Corgnac, S.; Pugnière, M.; Roquet, F.; Bascoul-Mollevi, C.; Baty, D.; Pèlegrin, A.; Navarro-Teulon, I. A llama single domain anti-idiotypic antibody mimicking HER2 as a vaccine: Immunogenicity and efficacy. *Vaccine.* **2009**, *27*, 4826-4833.
(16) Xiong, L.; Zhang, X.; Xu, Y.; Li, Y.; Liu, D.; Tu, Z.; He, Q. Anti-idiotypic VHH mediated environmentally friendly immunoassay for citrinin without mycotoxin. *Food and Agricultural Immunology*. **2020**, *31*, 968-984.
(17) Quintero-Campos, P.; Juarez, M. J.; Morais, S.; Maquieira, A. Multiparametric Highly Sensitive Chemiluminescence Immunoassay for Quantification of beta-Lactam-Specific Immunoglobulin E. *Anal Chem.* **2020**, *92*, 14608-14615.
(18) Tabares-da Rosa, S.; Rossotti, M.; Carleiza, C.; Carrion, F.; Pritsch, O.; Ahn, K. C.; Last, J. A.; Hammock, B. D.; Gonzalez-Sapienza, G. Competitive selection from single domain antibody libraries allows isolation of high-affinity antihapten antibodies that are not favored in the llama immune response. *Anal Chem.* **2011**, *83*, 7213-7220.
(19) Rossotti, M.; Tabares, S.; Alfaya, L.; Leizagoyen, C.; Moron, G.; Gonzalez-Sapienza, G. Streamlined method for parallel identification of single domain antibodies to membrane receptors on whole cells. *Biochim Biophys Acta.* **2015**, *1850*, 1397-1404.
(20) Olichon, A.; Schweizer, D.; Muyldermans, S.; de Marco, A. Heating as a rapid purification method for recovering correctly-folded thermotolerant VH and VHH domains. *BMC Biotechnol.* **2007**, *7*, 7.

Supporting information

Bispecific single domain antibodies as highly standardized synthetic calibrators for immunodiagnosis

Paula Segovia[1], Pedro Quinteros[2], Sergi Morais[2], Ángel Maquieira[2], Gabriel Lassabe[1*], Gualberto Gonzalez-Sapienza[1*].

[1]Cátedra de Inmunología, Facultad de Química, DEPBIO, Instituto de Higiene, Montevideo, Uruguay. [2]Departamento de Química, Universidad Politécnica de Valencia, Valencia, Spaín

Content

Figure S4. Preservation of the reactivity of the individual Nb components of the Az/Om calibrator.

Figure S5. Titration curves of the surrogate aztreonam IgE standard and the Az/Om calibrator.

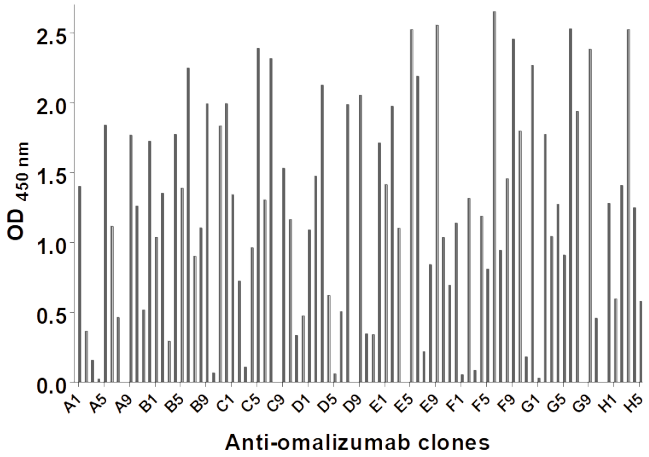


Fig. S1. Screening of clones expressing anti-omalizumab nanobodies. Reactivity of soluble cell extracts from bacterial cultures of 89 anti-omalizumab clones analyzed at a 10^{-7} dilution in ELISA plates coated with the omalizumab-KLH conjugate.

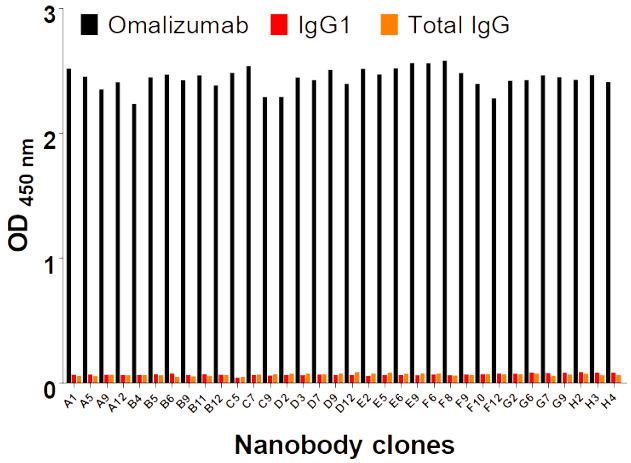


Fig. S2. Analysis of the specificity of the Nb clones against the paratope of omalizumab. Thirty four clones were analyzed by ELISA against omalizumab-KLH, human IgG1, and human total IgG.

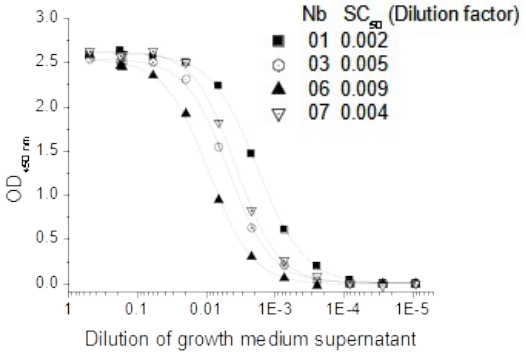
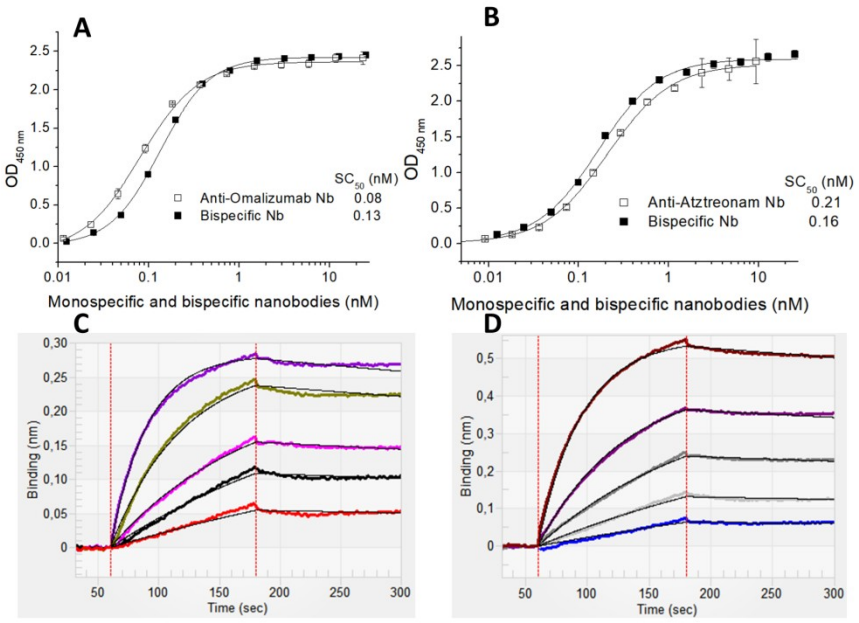


Fig. S3. Screening of clones expressing anti-Aztreonam nanobodies. Titration curves of bacterial culture supernatants in ELISA plates coated with aztreonam-KLH. The bound nanobody was detected with the anti-His-HRP conjugate.



	K_D (nM)	k_{on} (1/Ms)	k_{off} (1/s)
Nb E5	7.19	8.16×10^4	5.86×10^{-4}
Nb 01/Nb E5	6.97	6.96×10^4	4.85×10^{-4}

Figure S4. Preservation of the reactivity of the individual Nb components of the Az/Om calibrator. The antigenic reactivity of the bispecific Nb was compared to that of each of the monospecific Nbs. Titration curves were made by incubating serial dilutions of mono and bispecific nanobodies (triplicates) in ELISA plates coated with omalizumab (A), or KLH-aztreonam (B) Binding was detected with an anti His-tag antibody coupled to HRP. Binding of the mono (C) and bispecific (D) aztreonam Nb to omalizumab analyzed by biolayer interferometry (BLI). Bottom) Kinetic parameters of the BLI analysis.

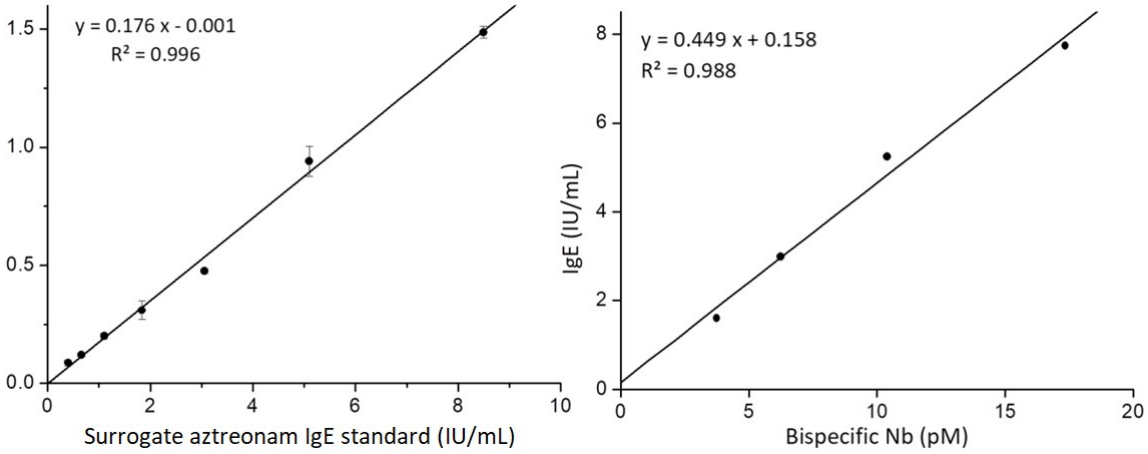


Figure S5. Titration curves of the surrogate aztreonam IgE standard (left) and the Az/Om calibrator (right). The curves were performed by ELISA on plates coated with human IgE.