Jackson ImmunoResearch LABORATORIES, INC.

INTRODUCING

Anti-Alpaca IgG, VHH domain **Secondary Antibodies**

PRODUCTS FOR DETECTION OF CAMELID VHH ANTIBODIES (NANOBODIES†)

Jackson ImmunoResearch's anti-alpaca secondary antibodies have been developed to optimize detection of antibodies derived from camelids (alpaca and llama).

INTRODUCING

Anti-Alpaca **Secondary Antibodies**

Heavy chain-only antibodies

VHH antibodies (nanobodies†) are rapidly being adopted in a variety of fields. Uses for these novel antibodies include various applications such as medical diagnostics, therapeutics, biosensors, crystallization partners, and detection of small molecules.

VHH antibodies are derived from the VHH domain of heavy chain-only immunoglobulins (IgG subclasses 2 and 3) from camelid species. VHH libraries derived from a camelid host animal (typically alpaca or llama) are screened for target specificity, and the desired VHH sequences are obtained. Recombinant VHH (rVHH) can then be produced in an expression system of choice. The long CDRs shown in the ribbon diagram (Figure 1) are characteristic of the VHH domain, and provide the antibody access to recessed antigenic sites not available to canonical antibodies.

Non-canonical immunoglobulins

Camelids (alpaca, llama, camel, vicuna and guanaco) produce two non-canonical immunoglobulins alongside the conventional IgG1. IgG2 and IgG3 are known as heavy chain-only (or heavy chain) antibodies. They have single domain binding sites (VHH), and do not contain CH1 domains or light chains (Figure 1).

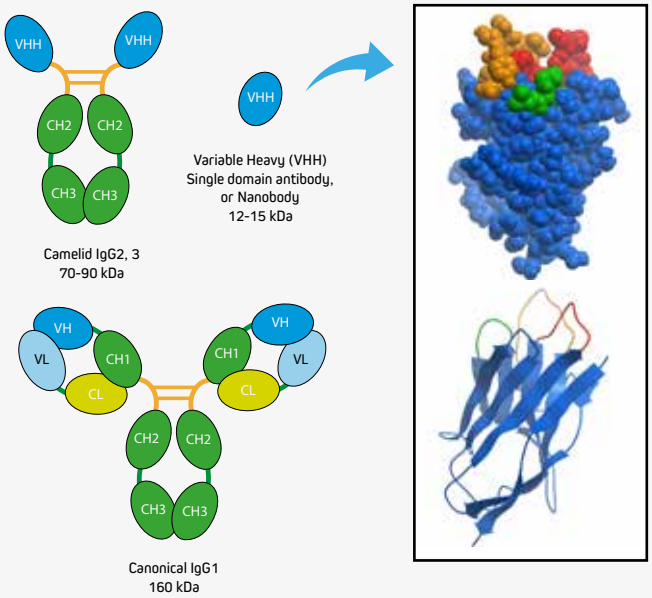

Figure 1: Conventional IgG, heavy chain-only IgG and VHH domain antibody. **Inset:** Space filling and ribbon diagram of the VHH domain antibody (PDB 1MEL) showing the 3 CDR loops, with the longer CDR3 loop shown in orange.

Benefits of VHH antibodies

Framework: The 12-15 kDa antigen-binding VHH domain acts as a framework for recombinant antibody production. VHH antibodies can be designed in a variety of structural formats.

Targeting: The small size and long CDR3 loop of the VHH antibodies allows better access to cryptic epitopes compared to conventional antibodies.

Stability: VHH antibodies have good solubility and are stable to heat, proteases and pH extremes.

Therapeutic advantages: VHH antibodies can cross the blood-brain barrier and access other remote locations, making them useful for drug delivery. They also clear quickly from circulation and are relatively non-immunogenic to humans (Bannas et al, 2017). Good stability allows delivery via a variety of routes, including intravenous and subcutaneous injection, nasal inhalation and oral ingestion.

Production: VHH antibodies are easy to clone and modify. They can be produced recombinantly at high levels in bacterial, yeast, and mammalian expression systems. Their design can include tags for purification, or functional groups to enable the addition of drugs or fluorescent probes.

Anti-VHH antibodies

Given their unique properties, VHH antibodies are growing in popularity as immunological tools. Jackson ImmunoResearch has created reagents to facilitate recognition of VHH antibodies in multiple platforms, including Western blotting, ELISA, flow cytometry, and immunofluorescence.

The utility of polyclonal secondary antibodies lies in their ability to recognize multiple epitopes on a target (primary) immunoglobulin. Anti-VHH domain antibodies are obtained by immunizing goats with alpaca VHH fragments which are highly purified from naïve alpaca serum. We have shown that the antibodies have robust recognition for both alpaca and llama VHH. However, some recombinant VHH may not display many of the antigens present on native VHH fragments, making these difficult to detect with the secondary antibodies.

Jackson ImmunoResearch offers two antibody specificities. AffiniPure Goat Anti-Alpaca IgG, VHH domain (min X Bovine Serum Proteins) (128-005-232) is broadly reactive, and is recommended for screening. For multiple labeling applications, or to minimize background tissue staining, use AffiniPure Goat Anti-Alpaca IgG, VHH domain (min X Bovine, Human, Mouse, Rabbit and Rat Serum Proteins) (128-005-230).

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Versatile detection of VHH antibodies regardless of species or format

Nanobodies are commonly generated from the VHH repertoire of either llama or alpaca heavy chain-only antibodies.

The Western blot (Figure 2) shows that Goat Anti-Alpaca VHH detects both alpaca and llama VHH frameworks in their denatured state. The variation in migration reflects differences among VHH antibody structures, notably the VHH dimer L4*.

As demonstrated by ELISA (Figure 3), Goat Anti-Alpaca VHH antibodies recognize the native form of both alpaca and llama rVHH. Different rVHH constructs are recognized with varying signal strength, suggesting that some rVHH do not express epitopes found on the immunogen (VHH fragments from naïve alpacas).

In most cases, 128-035-232 provides stronger signal than 128-035-230 because it is cross-adsorbed against fewer species. The intended application should be considered when choosing the antibody specificity: if species cross-reactivity is not a concern, use 128-035-232 for greater sensitivity.

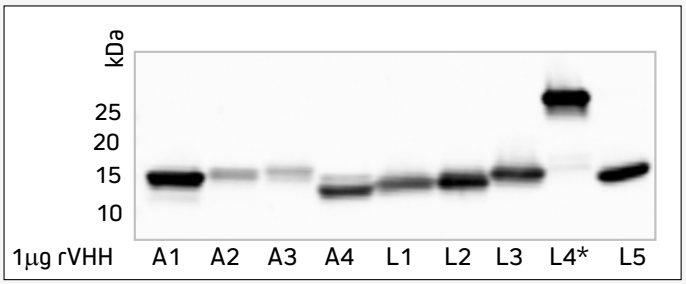


Figure 2: Western blot of different rVHH domain antibodies from both alpaca and llama. Purified rVHH (1µg/well) was run by PAGE under reducing conditions and transferred to nitrocellulose. Blots were blocked with BSA (001-000-162) and probed with HRP-conjugated Goat Anti-Alpaca VHH (128-035-

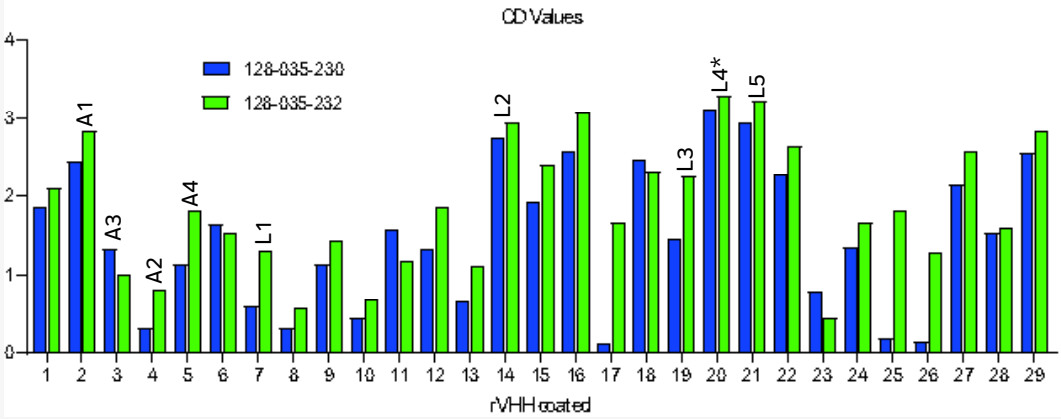


Figure 3: Detection of VHH antibodies from either alpaca or llama by ELISA. Each rVHH was coated onto the ELISA plate at 10 µg/ml and detected with HRP-conjugated Anti-Alpaca VHH (128-035-230 or 128-035-232) and TMB substrate.

Indirect detection of the protein of interest

After a VHH antibody has bound its target, detection using a secondary antibody is an alternative to tag-directed detection.

The secondary antibody provides signal amplification and the flexibility of increased conjugate options.

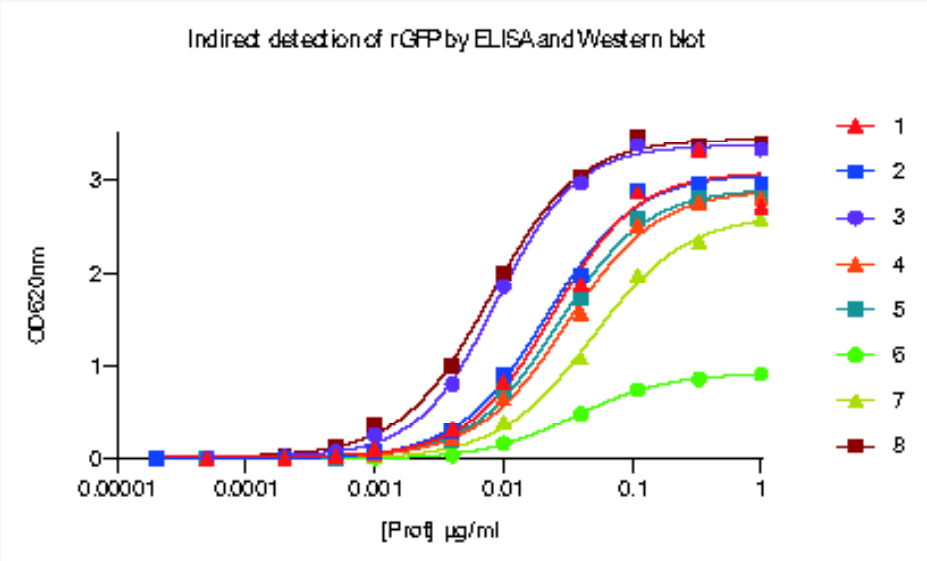


Figure 4: Detection of llama rVHH anti-GFP primary antibodies by ELISA. Purified recombinant GFP (rGFP) was coated onto the ELISA plate at 10 µg/ml. Wells were blocked with BSA (001-000-162) and probed with serial dilutions of several rVHH anti-GFP primary antibodies. Primary antibodies were detected with HRP-conjugated Goat Anti-Alpaca VHH (128-035-232) and TMB substrate.

Figure 4 inset: Western blot showing detection of a llama rVHH anti-GFP primary antibody. Two different purified rGFPs (0.5 µg/well) were run by PAGE under reducing conditions and transferred to nitrocellulose. Blots were blocked with BSA (001-000-162) and probed with rVHH anti-GFP primary antibody, followed by incubation with HRP-conjugated Goat Anti-Alpaca VHH (128-035-232) at a 1:10K dilution. ECL was used for detection.

Detection of VHH in biological samples

Detection may be required during the screening stage of VHH development, as well as in downstream applications. Direct detection using a secondary antibody specific for VHH, rather than through a tag added to the N- or C-terminus of the VHH, can offer advantages in many applications. For example, using anti-VHH domain antibodies circumvents detection through purification tags such as His6, reducing background from endogenous proteins which share homology with the tag epitope.

In Western blotting, Anti-Alpaca VHH antibodies enable targeted detection of rVHH in the presence of other proteins. When detecting recombinant protein from cell culture media or cell lysate, Anti-Alpaca VHH antibodies show significantly less non-specific detection when compared with anti-His6 antibodies (Figure 5).

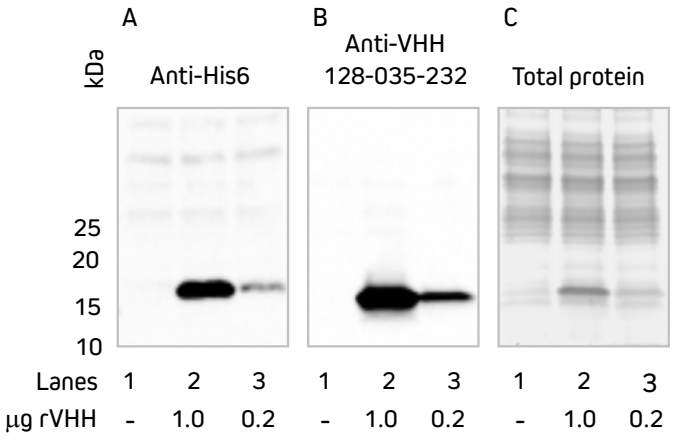
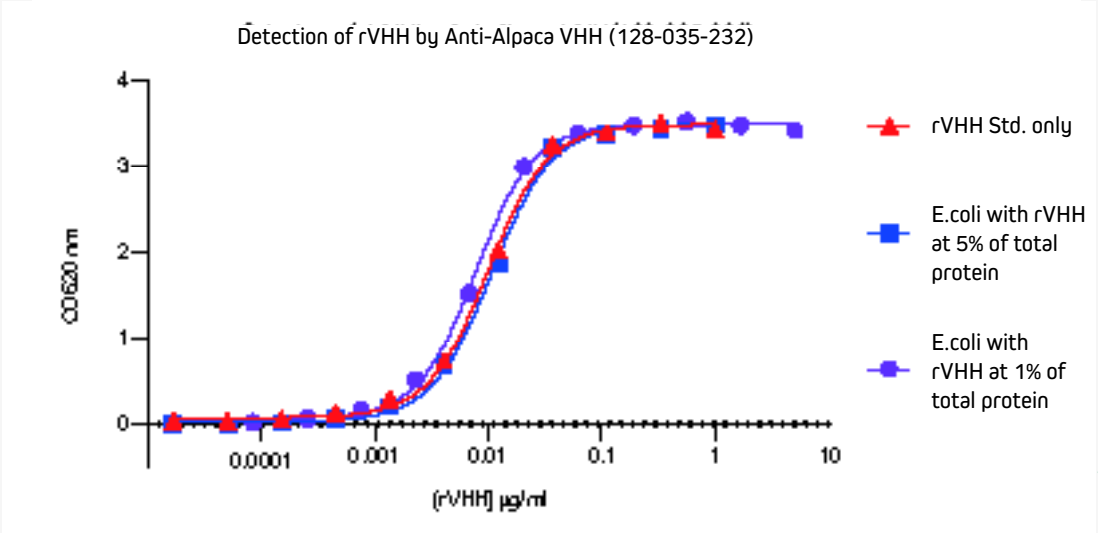


Figure 5: Western blot comparing detection of target protein in E. coli lysate. E. coli lysate proteins were separated by PAGE under reducing conditions and transferred to nitrocellulose. Blots were blocked with BSA (001-000-162) and probed with HRP-conjugated anti-His6 antibody or HRP-conjugated Goat Anti-Alpaca VHH (128-035-232) at a 1:5K dilution. ECL was used for detection, and blots were imaged simultaneously.

Lane 1. 20 µg E. coli extract only
Lane 2. 20 µg E.coli extract with rVHH expression at 5% of total protein (1 µg)
Lane 3. 20 µg E. coli extract with rVHH expression at 1% of total protein (0.2 µg)

Figure 6: Sandwich ELISA showing the detection of rVHH analyte in E. coli protein lysates. AffiniPure Goat Anti-Alpaca VHH (128-005-232) was coated onto ELISA plates as the capture antibody, followed by serial dilutions of a pure rVHH standard or E. coli lysates containing rVHH analyte at 1% or 5% of the total protein. rVHH was detected with HRP-conjugated Goat Anti-Alpaca VHH (128-035-232) and TMB substrate.



The ability to screen or quantify VHH expression prior to purification expedites discovery and production. However, the amount of VHH relative to total protein in cell lysate may be very small, making reliable characterization difficult. By targeting the VHH domain, Goat Anti-Alpaca IgG VHH domain antibodies remove the need for guesswork or tags. As shown in Figure 6, these polyclonal antibodies are able to detect VHH with high sensitivity and specificity in cell lysates.

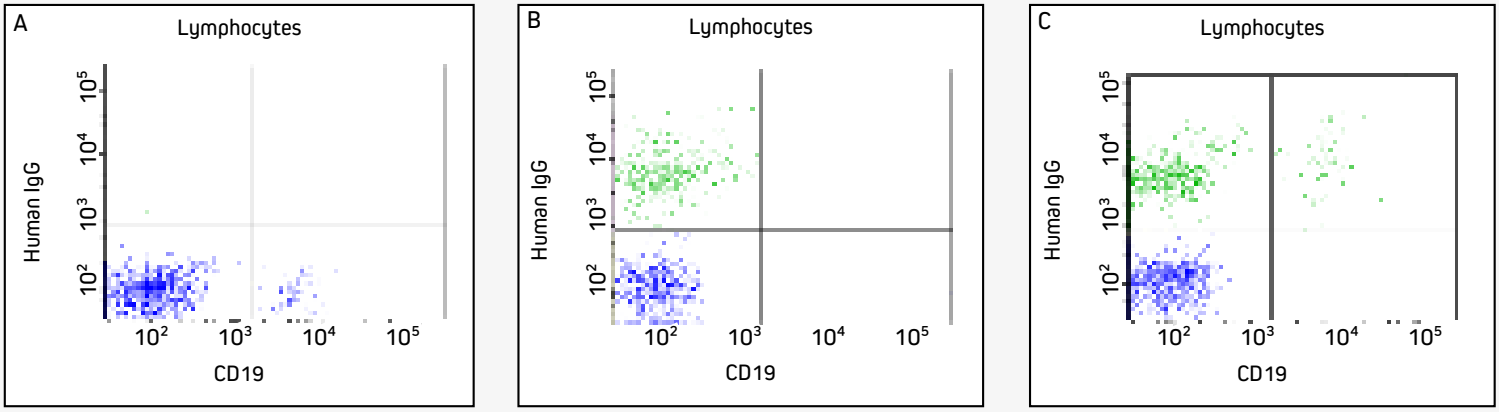
Detection of VHH by flow cytometry

Recombinant VHH constructs can be candidates for therapeutic applications, creating a need to track their presence in animal models or patients. Goat Anti-Alpaca VHH antibodies (e.g. Alexa Fluor® 488 conjugate 128-545-230) can be used to detect VHH on patient cells, without recognizing endogenous IgG or primary antibodies derived from mouse, rabbit or rat.

Current oncology research employs nanobody/VHH technology in a number of T cell redirection strategies to use (innate) immune defenses. These include the generation of bispecific formats to recruit and activate cytotoxic or γδ T cells, the design of nanobody-derived chimeric antigen receptor (CAR) T cells, and the development of immune checkpoint blocking nanobodies (Chanier and Chames, 2019). Characterization of the modified T cells is typically performed by flow cytometry, allowing sorting and expansion of the cells expressing the CAR.

We used polyclonal alpaca VHH anti-human IgG (rather than a CAR) to demonstrate the utility of 128-545-230 in flow cytometry. The panels below represent samples gated on lymphocytes. The Goat Anti-Alpaca VHH does not recognize the mouse anti-CD19, and detects human IgG only if the VHH anti-human IgG is present.

Figure 7: Indirect detection of Human IgG on lymphocytes using Alpaca VHH Anti-Human IgG and Alexa Fluor® 488 Goat Anti-Alpaca VHH



Panel A. Control, without VHH Anti-Human IgG primary antibody. Cells were incubated with Mouse anti-CD19 (APC-H7) and Alexa Fluor® 488 Goat Anti-Alpaca VHH (128-545-230) only. 128-545-230 does not recognize human IgG or the mouse primary antibody.

Panel B. Control, without Mouse anti-CD19. Cells were incubated with VHH Anti-Human IgG primary antibody and Alexa Fluor® 488 Goat Anti-Alpaca VHH only. Lymphocytes displaying IgG are detected.

Panel C. Double labeled cells. Cells were incubated with Mouse anti-CD19 (APC-H7), VHH Anti-Human IgG primary antibody, and Alexa Fluor® 488 Goat Anti-Alpaca VHH. Lymphocytes that are positive for both human IgG and CD19 are shown in the upper right quadrant.

Imaging with VHH antibodies

Detection using VHH antibodies requires reporter molecules such as fluorescent probes or enzymes to visualize the protein of interest. Conjugated Goat Anti-Alpaca VHH antibodies afford the inherent benefits of indirect detection such as signal amplification and conjugate choice.

Goat Anti-Alpaca VHH (min X Bov, Hu, Ms, Rb, Rat Sr Prot) allows detection of VHH antibodies while minimizing tissue background and mislabeling of primary antibodies from commonly used primary antibody hosts.

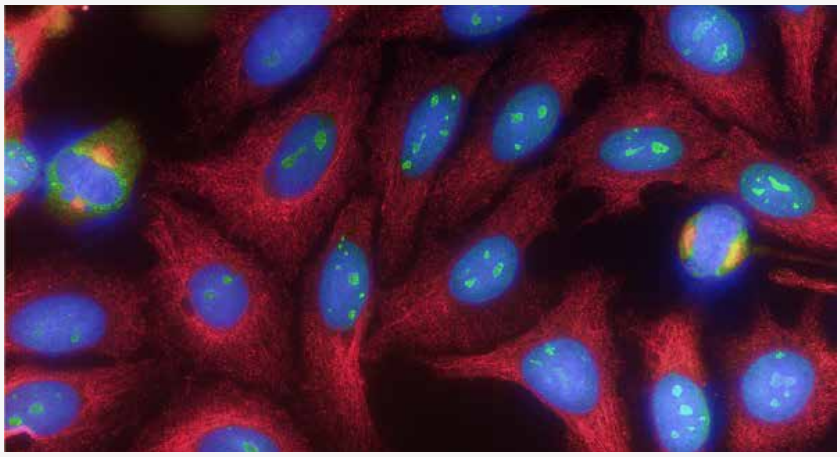


Figure 8: Double labeling of HEp-2 cells for Ki-67 and Tubulin.
HEp-2 cells were stained using the following combinations: Rabbit Anti-Ki-67, VHH Alpaca Anti-Rabbit (polyclonal), and Alexa Fluor® 488 conjugated Goat Anti-Alpaca IgG VHH domain (128-545-230) (green); Mouse Anti-Tubulin and RRX conj. Goat Anti-Mouse IgG (115-295-146) (red). Nuclear staining was performed using DAPI (blue).

SPECIALIZING IN

Secondary **Antibodies and Conjugates**

Jackson ImmunoResearch LABORATORIES, INC.

Anti-alpaca IgG secondary antibodies

Jackson ImmunoResearch's anti-alpaca secondary antibodies have been developed to optimize VHH antibody discovery at critical steps to ensure the generation of high-quality nanobody† candidates. JIR's anti-alpaca antibodies recognize both alpaca and llama proteins. Available antibody specificities are for IgG (H+L), IgG subclasses 2+3, or VHH domain.

AffiniPure Secondary Antibodies	Unconjugated
Goat Anti-Alpaca IgG (H+L)*	1.0 mg 128-005-003
Goat Anti-Alpaca IgG (H+L) (min X Bov, Hu, Ms, Rb, Rat Sr Prot)*	1.0 mg 128-005-160
Goat Anti-Alpaca IgG, subclasses 2+3 specific (min X Bov, Hu, Ms, Rb, Rat Sr Prot)*	0.5 mg 128-005-229
Goat Anti-Alpaca IgG, VHH domain (min X Bov Sr Prot)	0.25 mg 128-005-232
Goat Anti-Alpaca IgG, VHH domain (min X Bov, Hu, Ms, Rb, Rat Sr Prot)	0.25 mg 128-005-230

*These antibodies react primarily with the Fc region, and are not recommended for detection of VHH antibodies.

Available in a wide range of conjugate options

Horseradish Peroxidase	DyLight™ 405	Cy™ 3	Alexa Fluor® 594
Alkaline Phosphatase	Alexa Fluor® 488	R-Phycoerythrin (R-PE)	Alexa Fluor® 647
Biotin-SP	Fluorescein (FITC)	Rhodamine Red™-X	Cy™ 5

References:

Bannas, P., Hambach, J. and Koch-Nolte, F. (2017). Nanobodies and Nanobody-Based Human Heavy Chain Antibodies As Antitumor Therapeutics. Frontiers in Immunology, 8:1603.

Chanier, T. and Chames, P. (2019). Nanobody Engineering: Toward Next Generation Immunotherapies and Immunoimaging of Cancer. Antibodies, 8(1):13.

Sircar, A., Sanni, K., Shi, J. and Gray, J. (2011). Analysis and Modeling of the Variable Region of Camelid Single-Domain Antibodies. The Journal of Immunology, 186(11), pp.6357-6367.