



# Utilization of Nanobodies in Immunoturbidimetric Assays

Yue Lei Peng <sup>a</sup>, Hao Jie Han <sup>b</sup>, Bing Bing Du <sup>b</sup>,  
Run Ying Zeng <sup>b,c\*</sup> and Xin Yang <sup>a\*</sup>

<sup>a</sup> College of Life Sciences, Sichuan University, Chengdu-610065, China.

<sup>b</sup> Sjodax-TIO Union Laboratory of Marine Biogenetic Resources Development, Beijing, China.

<sup>c</sup> Third Institute of Oceanography, MNR, Xiamen, China.

## Authors' contributions

*This work was carried out in collaboration among all authors. Author YLP designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. "HaoJie Han, BingBing Du" managed the literature searches. Authors RYZ, XY managed the analyses of the study and revised the first draft of the manuscript. All authors read and approved the final manuscript.*

## Article Information

DOI: 10.9734/JABB/2023/v26i7646

## Open Peer Review History:

This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: <https://www.sdiarticle5.com/review-history/105775>

Original Research Article

Received: 02/07/2023

Accepted: 06/09/2023

Published: 07/09/2023

## ABSTRACT

**Aims:** Nanobodies, characterized by their small size, accessibility, and high yield, are emerging as advantageous tools in various applications. Despite these benefits, their integration into the formulation of immunoturbidimetric reagents has remained limited since their discovery.

**Methodology:** This study utilized nanobodies specific to cystatin C to formulate immunoturbidimetric reagents and compared them with traditional rabbit polyclonal antibody reagents.

**Results:** The findings revealed that the multiantibody affinity of Cys-rPAB surpasses that of Cys-VHH-104 and Cys-VHH-704 nanobodies, although the difference is not statistically significant. Upon evaluating the reagent formulation performance, both types of antibodies were found to satisfy the prerequisites of conventional immunoturbidimetric assay reagents. The correlation

\*Corresponding author: E-mail: yangxin0822@163.com, zeng@tio.org.cn;

coefficient R2 in the statistical analysis of the clinical results was 0.9986, signifying a high degree of consistency.

**Conclusion:** These findings underscore the feasibility and considerable economic potential of employing nanobodies in immunoturbidimetric reagents.

*Keywords: Nanobodies; immunoassay; affinity; cystatin C.*

## 1. INTRODUCTION

A specialized class of antibodies, referred to as nanobodies, has been sequentially discovered in two species, namely, camels and sharks. Professor Hamers-Casterman of the Free University of Brussels made the discovery in 1989 that antibodies derived from camels infected with *Trypanosoma evansi* were devoid of light chains. These antibodies, which lack light chains and are known as heavy-chain antibodies, are found within camelid species and are distinguished by the absence of the CH1 domain required for light chain pairing [1]. As a result, they contain only two heavy chains, with each chain encompassing a variable heavy chain domain (VHH) structural domain [2]. In 1998, Professor K.H. Roux uncovered novel immunoglobulin new antigen receptors (IgNAR) in nurse sharks, where the binding to antigens necessitates two independent, highly soluble new antigen receptors, of which the variable new antigen receptors (VNAR) is the smallest naturally occurring antibody-derived module, each with a molecular weight of approximately 12 kD [3]. With the progress of technology, researchers have elucidated the crystal structure of VHH/VNAR domains, with the VHH domain measuring approximately 2.5 × 4.0 nm and having a molecular weight of 15 kD (VNAR is 12 kD), as well as demonstrating the viability of selective identification, assessment, and recombinant expression of Nbs. This has contributed to an expanding application of nanobodies in various research fields [4,5].

In comparison to conventional monoclonal antibodies (mAb), nanobodies (Nbs) exhibit unique structural benefits. Their diminutive size, pronounced shape, and extensive complementarity-determining region 3 afford them specific complementary sites, allowing binding to certain obstructed antigenic recesses that are typically inaccessible to larger conventional monoclonal antibodies [6]. In the realm of disease diagnosis, Nbs have been widely employed in double-antibody sandwich ELISA methodologies to identify biomarker proteins, viruses, or other pathogens [7].

Immunoturbidimetry represents an immunological detection methodology deployed in precipitation reactions by synergizing contemporary optical measurement instruments with automated analytical systems. This technique enables the quantitative detection of a multitude of trace antigens, antibodies, drugs, and other small molecular semiantigens within bodily fluids [8]. Cystatin C, a 13 kD cysteine protease inhibitor protein, is synthesized at a constant rate by all nucleated cells, is freely filtered by the kidneys, and undergoes almost complete reabsorption and metabolic degradation within the proximal tubules, with negligible urinary excretion [9]. The concentration of this substance remains stable and uninfluenced by variables such as age, sex, muscle mass, the majority of medications, and inflammation [10]. Consequently, cystatin C can function as a renal performance metric and is poised to become a biomarker for glomerular filtration [11]. Since its inaugural detection via turbidimetry in 1994, cystatin C has evolved into an emblematic product in biochemical immunology, extending from ordinary turbidimetry to latex-enhanced turbidimetry [12].

In clinical practice, Cys-C testing reagents are largely reliant on rabbit polyclonal antibodies and have exhibited satisfactory performance, elevating the testing level from g/L to mg/L, with some latex turbidimetric reagents even reaching µg/L detection levels [12]. Nevertheless, given the utilization of Ig-class antibodies (inclusive of polyclonals and monoclonals), challenges such as elevated cost, interbatch variations (in the case of polyclonals), and interference from heterophilic antibodies persist, leading to suboptimal accuracy and occasional false positives in clinical assessments. The theoretical application of recombinant nanobodies may offer solutions to these issues.

At present, the market lacks immunoturbidimetric reagents crafted with Nbs antibodies. Within the context of this study, we isolated two strains of nanobodies from a previously established variable region library created with shark and camel nanobodies using the Cys-C antigen and

conducted recombinant expression. We subsequently contrasted these recombinant Nbs with rabbit polyclonal antibodies concerning antigen affinity and various performance indicators, including calibration, clinical correlation, and precision, when formulated as Cys-C immunoturbidimetric assay kits. This investigation assesses the prospective application of Nbs in in vitro diagnostic reagents and lays a scientific foundation for the innovation of new Cys-C immunoturbidimetric testing kits.

## 2. MATERIALS AND METHODS

### 2.1 Materials

The Cys-VHH104 nanobody, Cys-VHH704 nanobody, Cys-rPAB antibody, and Cys-C antigen were synthesized and prepared in our laboratory. JSR Corporation, Japan, supplied 10% 120 nm polystyrene microspheres. The 1x PBS was obtained from Pall Fortebio Company. Biotin at a concentration of 5 mg/ml was procured from Thermo Company. Additionally, disposable PD-10 Sephadex G-25 M columns and Greiner 96-well black microplates were furnished by Pall Fortebio Company. Beijing Kangwei Century Biotechnology Co., Ltd., provided the HRP-labeled anti-His tag rabbit polyclonal antibody.

### 2.2 Main Instruments and Reagents

Octet RED96 was supplied by Pall Fortebio Company, as was the SA sensor (lot: 1305151). The Duoflow was provided by Bio-Rad, while the enzyme immunoassay instrument was furnished by Thermo, and the biochemical analyzer was furnished by Toshiba. Latex reagents were prepared in accordance with the company's standard procedures, and the nanobody latex reagent was specifically formulated with the addition of 2.5% PEG6000 to augment the immune response.

### 2.3 Experimental Methods

#### 2.3.1 SDS-PAGE gel electrophoresis identification of antibodies

The purity and size of the recombinantly expressed and purified nanobodies and rabbit polyclonal antibodies were identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### 2.3.2 ELISA detection of antibody affinity

Cys-C antigen at varying dilutions (100X, 200X, 400X, 800X, 1600X, 3200X, 6400X, 12800X)

(original concentration: 1 mg/ml) was coated, followed by incubation with corresponding concentrations of antibodies. HRP-labeled secondary antibodies were incubated at 37°C for 40 min, followed by OPD color development, and the OD490 value was measured.

#### 2.3.3 Biolayer interferometry (BLI) measurement of antibody affinity

Using AntiBiotin chips, nanobody Cys-VHH and polyclonal Cys-rPAB were captured. Captured antibodies were then used to bind different concentrations of antigen, followed by dissociation in buffer. Finally, the affinity kinetic constants were calculated through instrument algorithms.

#### 2.3.4 Reagent preparation and determination of calibration curve

Nanobody Cys-VHH and rabbit polyclonal Cys-rPAB were added to our in-house latex reagent. The latex reagent was prepared according to the company's standard procedure, with 2.5% PEG6000 added to the nanobody reagent to enhance the immune response. Clinical testing was then conducted.

Calibrators of Cys-C were prepared at 0, 0.5, 1, 2, 4, and 8 mg/L by dilution. Absorbance was measured after reaction with both nanobodies and polyclonal antibodies, followed by color development, and the change in absorbance was measured. A linear curve was plotted with calibrator concentration as the x-axis and the change in absorbance as the y-axis, and the correlation coefficient curve  $r$  was calculated.

#### 2.3.5 Clinical relevance experiment

Thirty random clinical samples were tested with both reagents, and the results were statistically analyzed.

#### 2.3.6 Precision measurement of reagents

High and low concentrations of two clinical samples were selected. Ten repeated measurements were conducted using both VHH nanobodies and polyclonal reagents, and the average value, standard deviation, and coefficient of variation (CV) were calculated.

## 3. RESULTS AND DISCUSSION

### 3.1 Preparation and Detection of Cys-VHH-104/704 Nanobodies

The paired nanobodies Cys-VHH-104 and Cys-VHH-704, prepared in our laboratory through

expression in *Escherichia coli*, were verified alongside rabbit polyclonal Cys-rPAB using the SDS-PAGE method. The results exhibited distinct bands at the 20 kD mark for the nanobodies and corresponding bands of appropriate size for the polyclonals, indicating good purity of the antibodies that meet the requirements for subsequent experimental needs.

### 3.2 Measurement of Nanobody Affinity

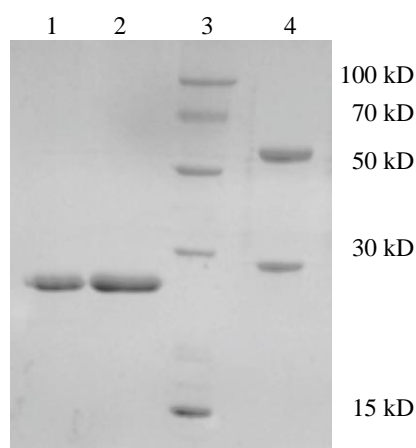
#### 3.2.1 ELISA detection of affinity between different antibodies and antigens

The affinity between Cys-VHH-104 and Cys-VHH-704 antibodies and Cys was assessed using the ELISA method. The experimental results (Table 1) revealed the order of affinity strength as follows: Cys-rPAB > Cys-VHH-104 > Cys-VHH-704. This indicates that polyclonal

antibodies indeed have their own advantages in terms of affinity, but the affinity of the nanobody Cys-VHH-104 is very close to that of Cys-rPAB.

#### 3.2.2 Comparison of nanobody and rabbit polyclonal antibody affinity for antigens through BLI experiments

By utilizing BLI to determine the relative affinities between different antibodies and their antigens, the results reveal that the affinity between antibody Cys-VHH-104 and antigen is 9.1 pM, while the average affinity between antibody Cys-VHH-704 and antigen is approximately 528.8 pM (550 pM & 507.7 pM). The affinity between the antibody Cys-rPAB and antigen is less than 1 pM. In summary, the ranking of the affinities between the three antibodies and their respective antigens is as follows: Cys-rPAB > Cys-VHH-104 > Cys-VHH-704, which aligns with the previous ELISA findings.



**Fig. 1. Validation of recombinant antibodies**

Note : 1. Cys-VHH-104; 2. Cys-VHH-704; 3. Marker; 4. Cys-rPAB

**Table 1. Protein titer determination results**

Dilution factor	Group			
	Cys-VHH-104	Cys-VHH-704	Cys-rPAB	Control group
100	2.984	2.772	3.012	0.111
200	3.039	1.407**	3.245	0.079
400	2.874	0.290	3.056	0.087
800	1.553*	0.137	2.587	0.082
1600	0.362	0.123	1.684***	0.085
3200	0.285	0.153	1.244	0.074
6400	0.019	0.124	0.524	0.084
12800	0.225	0.093	0.223	0.083

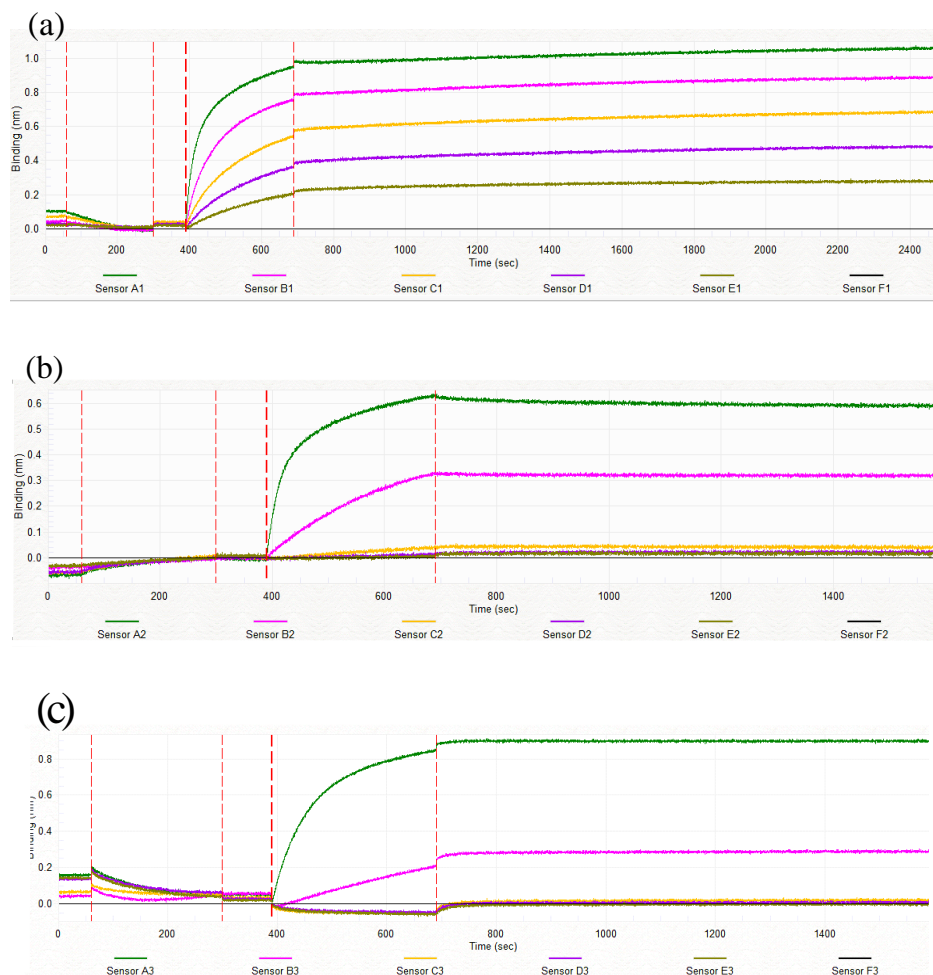
\* refers to the OD value corresponding to the antigen's respective dilution factor when Cys-VHH-104 reaches 1/2 OD

\*\* refers to the OD value corresponding to the antigen's respective dilution factor when Cys-VHH-704 reaches 1/2 OD

\*\*\*refers to the OD value corresponding to the antigen's respective dilution factor when Cys-rPAB reaches 1/2 OD

**Table 2. Statistical results of protein potency**

Antibody	Initial protein concentration	Protein concentration required to achieve 1/2 OD
Cys-VHH-104	0.73 mg/ml	0.59µg/ml
Cys-VHH-704	0.80 mg/ml	2.84µg/ml
Cys-rPAB	0.90 mg/ml	0.21µg/ml



**Fig. 2. Determination of antibody affinity**

a) Results of the Cys-rPAB Cys antibody; b) Results of the Cys-VHH-104 antibody; c) Cys-VHH-704 antibody

### 3.3 Reagent Preparation and Clinical Testing

#### 3.3.1 Reagent preparation and calibration curve

The nanobody Cys-VHH and rabbit polyclonal antibody Cys-rPAB were individually incorporated into our custom-made latex reagent, followed by clinical testing. Analyzing the results, the calibration sensitivity of the rabbit polyclonal antibody Cys-rPAB appears to be superior to that

of the nanobody Cys-VHH. However, both antibodies meet the requirements for normal immunoturbidimetric testing reagents.

#### 3.3.2 Clinical correlation analysis

A statistical correlation analysis was conducted on the clinical results obtained from the reagents containing the two different antibodies. The correlation coefficient R<sup>2</sup> was 0.9986, and the calculated results of the regression equation were found to be satisfactory.

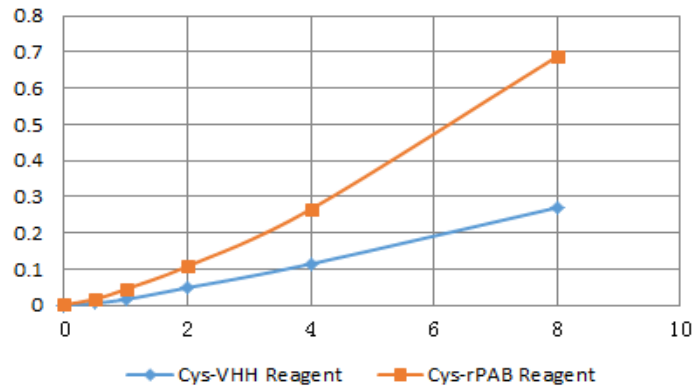


Fig. 3. Calibration Curve

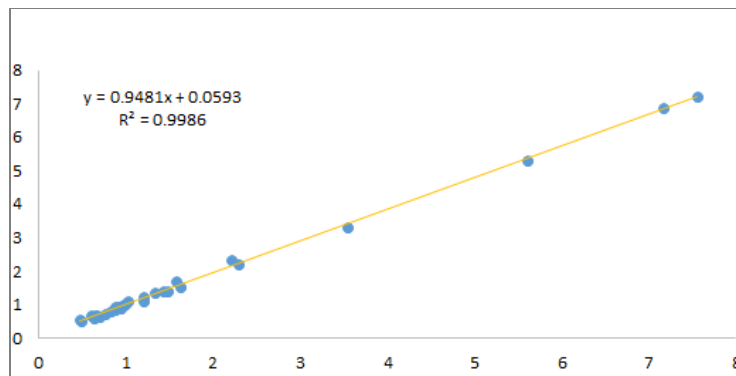


Fig. 4. Clinical correlation analysis

Table 3. Reagent precision testing

Group		Detection Indicators		
		AV*	SD*	CV* (%)
High value samples	Cys-VHH Reagent	0.757	0.021	2.79
	Cys-rPAB Reagent	0.739	0.019	2.59
Low value samples	Cys-VHH Reagent	3.32	0.028	0.84
	Cys-rPAB Reagent	3.135	0.022	0.69

\* AV: average value, SD: standard deviation, CV: coefficient of variation

### 3.3.3 Reagent precision testing

The measured values are shown in the Fig. 4. For the Cys-VHH reagent, the coefficients of variation for the high- and low-value samples were 2.79% and 0.84%, respectively. For the Cys-rPAB reagent, the CVs for the high- and low-value samples were 2.59% and 0.69%, respectively.

## 3.4 Discussion

Nbs are garnering attention in disease detection due to their ease of accessibility, cost-effectiveness, and high expression levels. For

example, in the diagnosis of SARS-CoV-2, Nbs are particularly suitable for binding to hidden or obscured epitopes, given their small size, making them a common choice for lateral flow tests [13]. Gai et al. isolated peripheral blood mononuclear cells from immunized camelids for VHH library construction and screening. The selected constructs were then expressed in a suitable vector, conjugated with gold nanoparticles (AuNPs), and employed in both ELISA and lateral flow tests [14]. Initially, when recombinant antibody engineering technology emerged, with a focus mainly on scFvs, Nbs did not gain much recognition. However, accumulating research has underscored their advantages in disease

detection studies [15]. In addition to the aforementioned disease detection applications, Nbs can retain functional stability under harsh chemical conditions, allowing the detection of food contaminants that induce protein denaturation through high-concentration methanol extraction [16,17].

The commercial industry has not yet extensively utilized Nbs antibodies in turbidimetric immunoassay reagents, prompting this exploratory study on the application of recombinant VHH domains in turbidimetric assays. Compared to conventional antibodies, Nbs, with their smaller size and correspondingly diminished antigen recognition sites, can access areas unreachable to traditional antibodies, showing considerable potential, especially in the detection of small molecule antigens. Presently, the detection of various vitamins, residual pesticides, narcotics, and additives still heavily depends on traditional methods such as MS or HPLC, which are characterized by low throughput and slow pace [18]. By employing Nbs in turbidimetric reagents, rapid and scalable detection becomes feasible, broadening the spectrum of testing scenarios. Although the unique structure of Nbs results in affinity comparatively lower than conventional Ig structure antibodies, this study demonstrated that CYS Nbs affinity can reach 10-12 levels, an improvement over the affinities of nanobodies such as 3Nb3 (3.6 nM) and those obtained through the ADAPT platform (2 nM) [19,20]. In clinical testing of the prepared Nbs turbidimetric reagent, the precision for both low and high concentration samples was comparable to rabbit polyclonal antibody reagents, with a significant reduction in the coefficient of variation compared to conventional polyclonal antibody kits [21]. Furthermore, the correlation coefficient R<sup>2</sup> of 0.9986 in clinical detection analysis aligns closely with previously reported values [22], substantiating the viability of Nbs application in biochemical immunoturbidimetry.

Cys Nbs can be expressed in *E. coli*, and the production efficiency is very high and the cost is greatly reduced. The cost of common mouse hybridoma monoclonal antibody or rabbit polyclonal antibody can be less than 10%, and the economic benefit is obvious. Secondly, genetically engineered recombinant mabs are easier to quality control in production and reduce the inter-batch variation. In summary, Cys Nbs recombinant monoclonal antibodies have significant economic and technological

advantages in detecting reagents. Based on Cys single resistance high affinity to prove that nano single resistance should be in pathological diagnosis and other application scenario has a high.

#### 4. CONCLUSION

This investigation elucidates that Nbs can function as a primary component in biochemical immunoturbidimetric reagents. Although its affinity falls short of rabbit polyclonal antibodies (10-13-10-12), it achieves the pM levels typical of mouse monoclonal antibodies. In alignment with calibration curves, clinical relevance, and precision standards, it fulfills the criteria for conventional immunoturbidimetric detection reagents, indicating that Nbs holds substantial prospective applicative value in biochemical reagent preparation.

#### ACKNOWLEDGEMENTS

A brief acknowledgement section may be given after the conclusion section just before the references. The acknowledgments of people who provided assistance in manuscript preparation, funding for research, etc. should be listed in this section. All sources of funding should be declared as an acknowledgement. Authors should declare the role of funding agency, if any, in the study design, collection, analysis and interpretation of data; in the writing of the manuscript. If the study sponsors had no such involvement, the authors should so state.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

#### REFERENCES

1. Muyldermans S. Applications of nanobodies. *Annu Rev Anim Biosci.* 2021; 16;9:401-421. DOI: 10.1146/annurev-animal-021419-083831. Epub 2020 Nov 24. PMID: 33233943
2. Ingram JR, Schmidt FI, Ploegh HL. Exploiting nanobodies' singular traits. *Annu Rev Immunol.* 2018;26:36:695-715. DOI: 10.1146/annurev-immunol-042617-053327. Epub 2018 Feb 28. PMID: 29490163
3. Khalid Z, Chen Y, Yu D, Abbas M, Huan M, Naz Z, Mengist HM, Cao MJ, Jin T. IgNAR antibody: Structural features,

- diversity and applications. Fish Shellfish Immunol. 2022;121:467-477.  
DOI: 10.1016/j.fsi.2022.01.027. Epub 2022 Jan 22. PMID: 35077867..
4. De Groeve M, Laukens B, Schotte P. Optimizing expression of Nanobody® molecules in *Pichia pastoris* through co-expression of auxiliary proteins under methanol and methanol-free conditions. Microb Cell Fact. 2023;22:22(1):135.  
DOI: 10.1186/s12934-023-02132-z. PMID: 37481525; PMCID: PMC10362571
  5. Juma SN, Gong X, Hu S, Lv Z, Shao J, Liu L, Chen G. Shark New Antigen Receptor (IgNAR): Structure, characteristics and potential biomedical applications. Cells. 2021;10(5):1140.  
DOI: 10.3390/cells10051140. PMID: 34066890; PMCID: PMC8151367
  6. Khodabakhsh F, Behdani M, Rami A, Kazemi-Lomedasht F. Single-domain antibodies or nanobodies: A class of next-generation antibodies. Int. Rev. Immunol. 2018;37:316–322.  
DOI: 10.1080/08830185.2018.1526932.
  7. Verhaar ER, Woodham AW, Ploegh HL. Nanobodies in cancer. Semin Immunol. 2021;52:101425.  
DOI: 10.1016/j.smim.2020.101425. Epub 2020 Nov 30. PMID: 33272897; PMCID: PMC8164649.
  8. Yang X, Shu W, Wang Y, Gong Y, Gong C, Chen Q, Tan X, Peng GD, Fan X, Rao YJ. Turbidimetric inhibition immunoassay revisited to enhance its sensitivity via an optofluidic laser. Biosens Bioelectron. 2019;15:131:60-66.  
DOI: 10.1016/j.bios.2019.02.013. Epub 2019 Feb 19. PMID: 30826651
  9. Zhang J-r, Wang Y, Dong J-x, Yang J-y, Zhang Y-q et al. Development of a simple pretreatment immunoassay based on an organic solvent-tolerant nanobody for the detection of carbofuran in vegetable and fruit samples. Biomolecules 2019;9(10): 576.
  10. Yang H, Lin C, Zhuang C, Chen J, Jia Y, Shi H, Zhuang C. Serum Cystatin C as a predictor of acute kidney injury in neonates: a meta-analysis. J Pediatr (Rio J). 2022;98(3):230-240.  
DOI: 10.1016/j.jpmed.2021.08.005. Epub 2021 Oct 16. PMID: 34662539; PMCID: PMC9432009..
  11. Liao X, Zhu Y, Xue C. Diagnostic value of serum cystatin C for diabetic nephropathy: A meta-analysis. BMC Endocr Disord. 2022;22(1):149.  
DOI: 10.1186/s12902-022-01052-0. PMID: 35655297; PMCID: PMC9164876..
  12. Benoit SW, Ciccia EA, Devarajan P. Cystatin C as a biomarker of chronic kidney disease: Latest developments. Expert Rev Mol Diagn. 2020;20(10):1019-1026.  
DOI: 10.1080/14737159.2020.1768849. Epub 2020 May 25. PMID: 32450046; PMCID: PMC7657956.
  13. Pymm P, Adair A, Chan LJ, Cooney JP, Mordant FL, Allison CC, Lopez E, Haycroft ER, O'Neill MT, Tan LL, Dietrich MH, Drew D, Doerflinger M, Dengler MA, Scott NE, Wheatley AK, Gherardin NA., Venugopal H, Cromer D, Davenport MP, Pickering R, Godfrey DI, Purcell DFJ, Kent SJ, Chung AW., Subbarao K, Pellegrini M, Glukhova A, Tham W.-H. Nanobody cocktails potently neutralize SARS-CoV-2 D614G N501Y variant and protect mice. Proc. Natl. Acad. Sci. 2021;118.  
DOI: 10.1073/PNAS.2101918118.
  14. Gai J, Ma L, Li G, Zhu M, Qiao P, Li X, Zhang H, Zhang Y, Chen Y, Gong R, Wan Y. A potent neutralizing nanobody against SARS-CoV-2 with inhaled delivery potential. MedComm. 2021;2:101–113.  
DOI: 10.1101/2020.08.09.242867
  15. Aguilar G, Matsuda S, Vigano MA, Affolter M. Using nanobodies to study protein function in developing organisms. antibodies (Basel). 2019;12;8(1):16.  
DOI: 10.3390/antib8010016. PMID: 31544822; PMCID: PMC6640693.
  16. He J, Ma S, Wu S, Xu J, Tian J, Li J, Gee S, Hammock B, Li Q, Xu T. Construction of immunomagnetic particles with high stability in stringent conditions by site-directed immobilization of multivalent nanobodies onto bacterial magnetic particles for the environmental detection of tetrabromobisphenol-A. Anal Chem. 2020; 92:1114–1121.
  17. Muyldermans S. A guide to: Generation and design of nanobodies. FEBS J. 2021;288(7):2084-2102.  
DOI: 10.1111/febs.15515. Epub 2020 Aug 28. PMID: 32780549; PMCID: PMC8048825.
  18. Zheng K, Lin R, Liu X, Wu X, Chen R, Yang M. Multiresidue pesticide analysis in tea using GC-MS/MS to determine 12



- pesticide residues (GB 2763-2021). *Molecules*. 2022;1;27(23):8419. DOI: 10.3390/molecules27238419. PMID: 36500512; PMCID: PMC9735578.
19. Baharlou R, Tajik N, Habibi-Anbouhi M et al. Generation and characterization of an anti-delta like ligand-4 Nanobody to induce non-productive angiogenesis [J]. *Anal Biochem*. 2018;544:34-41.
  20. Vivcharuk V, Baardsnes J, Deprez C, et al. Assisted design of antibody and protein therapeutics (ADAPT) [J]. *PLOS ONE*. 2017;12(7):0181490.
  21. Natarajan Satheesh, DeRosa Maria C, Shah Malay Ilesh, Jayaraj Joseph. Development and Evaluation of a Quantitative Fluorescent Lateral Flow Immunoassay for Cystatin-C, a Renal Dysfunction Biomarker. *Sensors (Basel)*. 2021;21(9). (Undefined). DOI:10.3390/s21093178
  22. Gao Yang, Guo Yuguang, Hao Wenjun., Meng Jin, Miao Zhilin, Hou Aijie, Luan Bo. Correlation Analysis and Diagnostic Value of Serum Homocysteine, Cystatin C and Uric Acid Levels with the Severity of Coronary Artery Stenosis in Patients with Coronary Heart Disease. *Int J Gen Med*. 2023; 16(undefined): 2719-2731. DOI:10.2147/IJGM.S411417

© 2023 Peng et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

*Peer-review history:*  
The peer review history for this paper can be accessed here:  
<https://www.sdiarticle5.com/review-history/105775>