Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

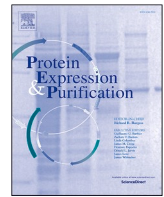
Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Protein Expression and Purification 194 (2022) 106071

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



Expression, purification and characterization of SARS-CoV-2 spike RBD in ExpiCHO cells

Matteo De March [a,b,*], Michela Terdoslavich [b], Sulena Polez [b], Corrado Guarnaccia [b], Monica Poggianella [c], Alessandro Marcello [c], Nataša Skoko [b], Ario de Marco [a,**]

[a] Laboratory for Environmental and Life Sciences, University of Nova Gorica, Vipavska cesta 13, SI-5000, Nova Gorica, Slovenia
[b] Biotechnology Development Unit, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149, Trieste, Italy
[c] Laboratory of Molecular Virology, International Centre for Genetic Engineering and Biotechnology, Padriciano 99, 34149, Trieste, Italy

ARTICLE INFO

Keywords:
Antibody binding assay
ExpiCHO cells
Protein production
Receptor binding domain (RBD)
SARS-CoV-2 Spike

ABSTRACT

Reliable diagnosis is critical to identify infections of SARS-CoV-2 as well as to evaluate the immune response to virus and vaccines. Consequently, it becomes crucial the isolation of sensitive antibodies to use as immunocapture elements of diagnostic tools. The final bottleneck to achieve these results is the availability of enough antigen of good quality. We have established a robust pipeline for the production of recombinant, functional SARS-CoV-2 Spike receptor binding domain (RBD) at high yield and low cost in culture flasks.

RBD was expressed in transiently transfected ExpiCHO cells at 32 °C and 5% CO_2 and purified up to 40 mg/L. The progressive protein accumulation in the culture medium was monitored with an immunobinding assay in order to identify the optimal collection time. Successively, a two-step chromatographic protocol enabled its selective purification in the monomeric state. RBD quality assessment was positively evaluated by SDS-PAGE, Western Blotting and Mass Spectrometry, while Bio-Layer Interferometry, flow cytometer and ELISA tests confirmed its functionality. This effective protocol for the RBD production in transient eukaryotic system can be immediately extended to the production of RBD mutants.

1. Introduction

The clinical, social and economic impact of the global pandemic caused by coronavirus disease 2019 (COVID-19) has promoted a global effort to investigate any aspect related to the biology of severe-acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. SARS-CoV-2 entry into the host cell is primarily driven by the receptor-binding domain (RBD) of Spike glycoprotein S that forms a homotrimer able to interact with the host cell receptor angiotensin-converting enzyme 2 (ACE2) [2–5]. Because of its role, RBD is also the major target of neutralizing antibodies [6–8], both natural and recombinant [9].

The accurate detection of Spike RBD in biological samples is critical to infer the presence of virus particles and monitor vaccine efficiency [10]. The diagnostic trend moves toward rapid and not invasive portable tests that can exploit alternative fluids (sputum, blood and urine) in standardized procedures simpler than processing swabs used to isolate virus particles in the higher respiratory tract [11]. Miniaturized immunobiosensors functionalized with antibody fragments have the features to become inexpensive point-of-care diagnostic tools [9,12,13] and ligands as adhirons (affimers) [14] might represent an interesting alternative because of their biophysical features. With the perspective of selecting anti-RBD adhirons for a new biosensor, we envisaged a Spike RBD production protocol more effective than the strategies already available. If bacterial expression provides deglycosylated and mainly insoluble RBD [15–18] (Table 1), eukaryotic systems based on cells grown in both flasks and bioreactors were successful to express RBD [10, 19–23] but had specific shortcomings (Table 1). For instance, RBD production in yeast reached yields of 60 mg/L [22] but showed high level of immunogenic N-glycans which made it unsuitable for serological tests, whereas RBD expressed in insect cells was O-glycosylated [23] (Table 1). CHO cells are largely used for biotherapeutic protein expression and provide human-like post-translational modifications

Abbreviations: ExpiCHO, Experimental Chinese Hamster Ovary; HPLC, High Performance Liquid Chromatography; Ab, antibody; RBD, Receptor Binding Domain; ACE-2, Angiotensin converting enzyme 2; HEK, Human Embryonic Kidney.

* Corresponding author. Laboratory for Environmental and Life Sciences, University of Nova Gorica, Vipavska cesta 13, SI-5000, Nova Gorica, Slovenia.
** Corresponding author.
E-mail addresses: matteo.demarch@ung.si (M. De March), ario.demarco@ung.si (A. de Marco).

https://doi.org/10.1016/j.pep.2022.106071
Received 23 December 2021; Received in revised form 2 February 2022; Accepted 11 February 2022
Available online 13 February 2022
1046-5928/© 2022 Elsevier Inc. All rights reserved.

Table 1
Approaches used for the production of SARS-CoV-2 Spike RBD.The characteristics of the constructs are listed together with information about the expression system, the yield of purified protein, relevant annotations and literature references.

RBD	EXPRESSION SYSTEM	YIELD mg/L	Comments	REFERENCE
320–537	Mammalian CHO (stable)	30	31% dimer	Sinegubova et al., 2021
319-541 Krammer	Mammalian Expi293F (transient)	Not indicated		Tee et al., 2020
331–591	Mammalian HEK293SF (transient)	150	Bioreactor	Farnos et al., 2020
319–541	Baculovirus	30		Li et al., 2020
305–543	Insect cells ExpreS^2	Not indicated	O-glycosylated	Bagdonaite et al., 2021
318–536 N1	Yeast *P. pastoris* X-33	60	Bioreactor Hyperglycosylated	Chen et al., 2017
330–583	Bacteria *E. coli*	13.3	Deglycosylated	He et al., 2021
319–640	Bacteria *E. coli*	Not indicated	Deglycosylated and not properly functional	Fitzgerald et al., 2021
389–611	Bacteria *E. coli*	Not indicated	CRM197-RBD Refolded from inclusion bodies	Bellone et al., 2021
319–541	Bacteria *E. coli*	Not indicated	Refolded from inclusion bodies	Gao et al., 2021

necessary for the correct biological activity but almost 30% of RBD purified from stably transformed CHO cells was dimeric [10] (Table 1). Notably, CHO-DXB11 derived cells were successfully used to produce full length Spike ectodomain up to 100–150 mg/L [24].

Here we tested transiently transfected high density ExpiCHO cells [25] cultured in flasks for expressing Spike RBD and obtained monomeric and functional antigen through a fast and cost-effective protocol that can be immediately available for the production of RBD mutants.

2. Material and methods

The RBD sequence cloned in the pCAGGS vector was kindly provided by Krammer lab [26]. In this construct, the native N-terminal signal peptide of Spike protein (MFVFLVLLPLVSSQ) was fused to the RBD region (aa 319 to aa 541) and joined with a C-terminal 6xHis tag. ExpiCHO cells were thawed for 10 min and cultured for 6–10 days at 37 °C, 8% CO_2 and 120 rpm in 60 mL of ExpiCHO expression medium (pre-warmed at 37 °C) using Erlenmeyer flasks. Two to three passages of gently dead-cells filtration and media exchange were applied to maintain culture in the optimal growth conditions. Transfection was performed following the ExpiCHO™ Expression System Kit instructions (Thermo-Fisher - code A29133) without antibiotics and applying the Max titer protocol when cells reached Viable Cell Density (VCD) of 6×10^6 cells/mL and doubling time of 18–20 h. The pCAGGS-RBD plasmid (1.4 mg/mL and $A_{260/280} = 1.9$) was prepared using the PureLink™ Hipure Maxiprep Kit from Thermofisher (code K210006) to recover sterile, salt-free and supercoiled DNA. ExpiFectamine CHO was mixed by repeated inversions, diluted with cold OptiPRO medium and finally complexed to the diluted plasmid DNA at room temperature. After 5 min the mix was added drop by drop to the cell culture at room temperature.

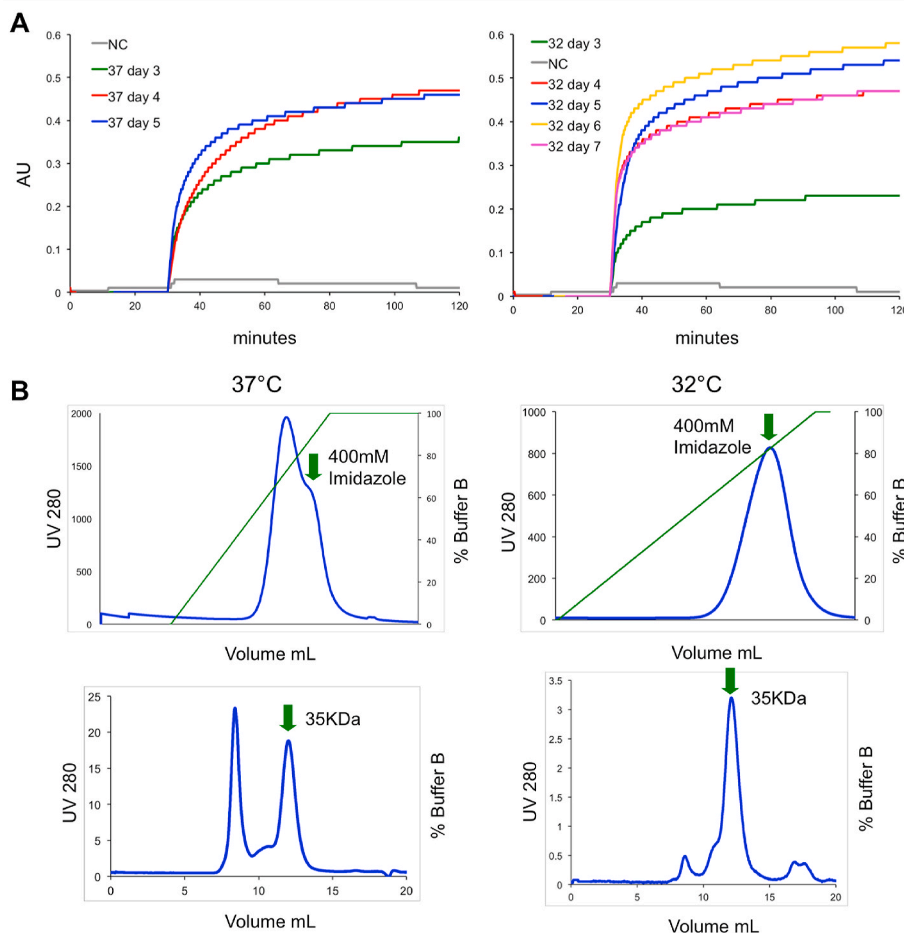


Fig. 1. Production of monomeric SARS-CoV-2 Spike RBD at 37 °C and 32 °C in ExpiCHO cells. **A.** Biolayer Interferometry protein-antigen binding assay performed with CR3022-AbSIP as ligand was used to follow the protein accumulation (AU, arbitrary unit) in cell cultures. Daily concentrations were compared for cells grown at both 37 °C and 32 °C. NC (negative control) corresponds to not transfected cells. **B.** IMAC-Affinity (top) and Gel Filtration (bottom) chromatographic steps of SARS-CoV-2 Spike RBD. Green arrows indicate the RBD elution peaks.

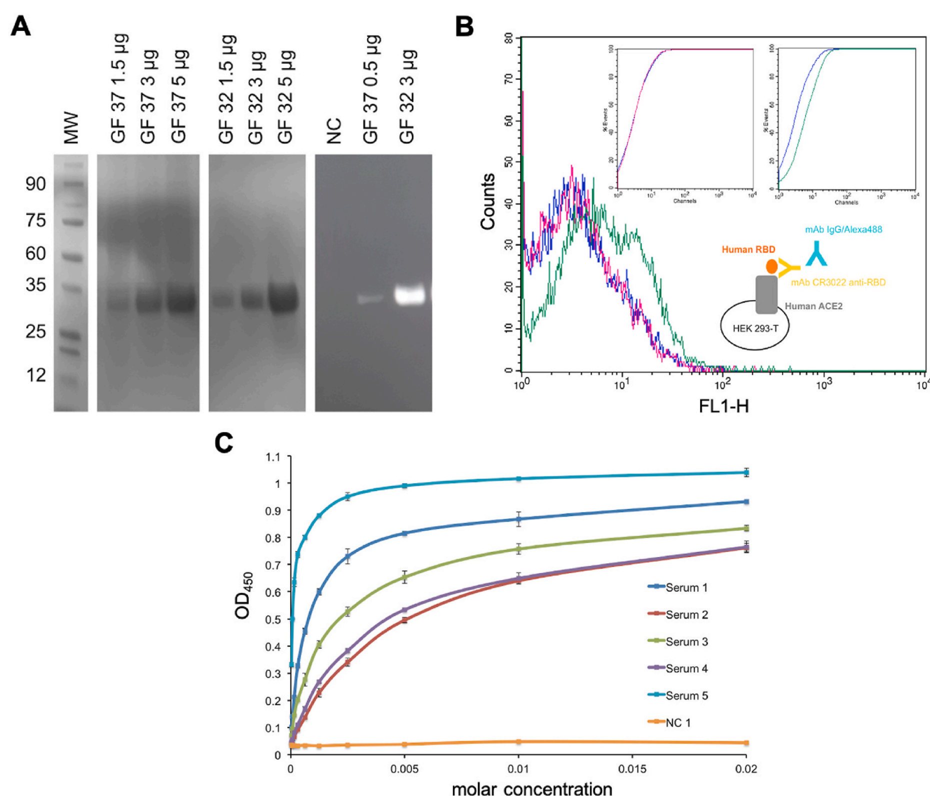


Fig. 2. Characterization of purified SARS-CoV-2 Spike RBD expressed at 37 °C and 32 °C in Expi-CHO cells. A. SDS-PAGE of RBD fractions after Gel Filtration chromatography (GF) and Western Blot visualized with commercial monoclonal anti-RBD antibody. B. Detection of RBD binding activity on human ACE2 receptor expressed in HEK-293T cells by flow cytometry. Green signal corresponds to cells expressing ACE2 interacting with RBD (20 μg/mL) and recognized by the primary mouse antibody CR3022-AbSIP (1 μg/mL) and the secondary anti Human IgG goat antibody alexa 488 (1:1000). Blue signal (unlabeled ACE2/HEK cells) and pink signal (cells expressing ACE2 bound to RBD with secondary mAb-Alexa antibody) are the blank and the negative control, respectively. C. ELISA assay for the detection of specific anti-SARS-CoV-2 RBD IgG antibodies in the sera of 5 patients pre (NC) and post (sera 1–5) infection by SARS-CoV-2 virus. For simplicity, only NC from patient 1 is represented. The OD_{450} of the 3,3′,5,5′-Tetramethylbenzidine substrate bound to the goat HRP-conjugate anti-Human IgG γ-chain antibody (1:5000) was monitored at 4 M dilutions.

For Biolayer Interferometry, ammine reactive second-generation sensors (AR2G) were irreversibly immobilized with the fusion protein composed by the CH2-CH3 domains of mouse immunoglobulin and the scFv domain of the anti-RBD human IgG1 CR3022 (hereafter CR3022-AbSIP) [27].

Culture media were centrifuged at 25 °C and 500×*g* for 5 min to pellet cells and then at 4 °C and 4500×*g* for 30 min. The clarified supernatant was collected and injected into a HiPrep™ 26/10 desalting column. This sample was then injected into a 1 mL HiTrap™ chelating HP Nickel column previously equilibrated in 20 mM $NaHPO_4$ pH 7.4, 500 mM NaCl, 10 mM imidazole. The gel filtration step was performed with a Superdex 75 10/300 (Cytiva) column equilibrated with 20 mM $NaHPO_4$ pH 7.4 and 100 mM NaCl. To avoid protein precipitation on nitrocellulose membranes observed in preliminary tests, we concentrated sample on 10 MWCO PES (polyethersulfone) Vivaspin filters (Sartorius) before quantification by UV absorbance at 280 nm.

RP-HPLC was run in an Agilent 1100 system thermostated at 25 °C, equipped with an analytical column Zorbax 300SB - C18 5 μm 4.6 × 250 mm and UV lamp set at 280 nm in combination with a 30 min acetonitrile gradient (0%–100%, flowrate of 1 mL/min). TFA (0.1% v/v) was added to the mobile phase.

Tandem Mass Spectrometry (MS/MS) was used applying multiple reaction monitoring of peptides after tryptic digestion [28]. Specifically, the sample was heated for 10 min at 99 °C in reducing buffer (0.4 M Tris/HCl pH 8, 200 mM DTT), then incubated first at room temperature for 10 min and then at 37 °C for 2 h in 0.1 M Tris/HCl and 1 μg of trypsin. After reaction quenching with 2% formic acid, the sample was injected into a HPLC Agilent 1260 linked to an ESI-IT Bruker AmaZon SL to identify the resulting RBD peptides. Peptide FASVYAWNR (aa 77–85) with the doubly charged ion at m/z 557.2 was monitored in fragments y4 (m/z 546.3) and y5 (m/z 709.3), while the peptide VGGNYNYLYR (aa 175–184) with a doubly charged ion at m/z 609.7 was monitored in the fragment y5 (m/z 728.3).

For flow cytometer analysis, HEK-293T cells expressing ACE2 receptor were incubated 1h at 4 °C in the presence of purified RBD, the primary mouse antibody CR3022-AbSIP and the secondary anti Human IgG goat antibody Alexa 488 [29]. 96-well ELISA Maxi Sorp Immuno-Plates (Thermofisher) were coated with SARS-CoV-2 RBD (1 μg/mL in PBS pH 7.4) and incubated overnight at 4 °C. Plates were then washed 3 times with PBS pH 7.4 and 0.05% Tween-20 (PBST) using the BioRad immune-plate washer and blocked with 3% milk-PBST. Serial molar dilutions of human sera in 1% milk-PBST were added to the plates and incubated for 2 h at RT. After washing, the goat HRP-conjugate anti-Human IgG γ-chain antibody was incubated for 1h at RT before the addition of 3,3′,5,5′-Tetramethylbenzidine substrate for 10 min at RT. Reactions were stopped by adding 50 μL/well of 1 M H_2SO_4. OD_{450} was measured on Bio-Rad iMark microplate reader.

3. Results and discussion

After transfection at day 1, the temperature was decreased to 32 °C and the CO_2 to 5% within 5 h with the addition of ExpiCHO feed and ExpiFectamine enhancer to the culture. In parallel, a second transfected culture was left at 37 °C and 8% CO_2 to evaluate the temperature effect on the RBD expression level. Starting from Day 3, Biolayer Interferometry (BLI, performed with BLItz, ForteBio) was used to monitor the RBD accumulation in the culture media and to identify the optimal time to stop expression. This corresponded to the moment in which the total cell number decreases of 30% with respect to the maximum and RBD accumulation reached the steady-state. Specifically, the optimal conditions were reached at day 5 and 7 for expression at 37 °C and at 32 °C, respectively (Fig. 1A).

From HiPrep™ 26/10 desalting column we got three peaks, with the first one corresponding to the RBD (Supplementary Fig. S1) that was eluted from a 1 mL HiTrap™ chelating HP Nickel column with an imidazole gradient between 400 mM and 500 mM at pH 7.0 (Fig. 1B). The protein expressed at 32 °C was eluted as a single peak, whereas the protein expressed at 37 °C co-eluted as a shoulder of the major peak that corresponded to an unrelated larger protein (Figs. 1B and 2A). RBD expressed at 32 °C eluted fully as a monomer from gel filtration step

performed with a Superdex 75 10/300, while RBD expressed at 37 °C was partially aggregated, as indicated by the presence of an early peak in Fig. 1B. The single band of 35 kDa in the SDS-PAGE (Fig. 2A) and the specific binding to the mouse mAb anti-RBD (clone# 1034522, R&D) in Western Blot confirmed the protein purity and epitope conservation (Fig. 2A). No protein degradation was detectable in both preparations after purification. The purification of RBD expressed in ExpiCHO cells at 37 °C produced 6 mg/L. This method was dismissed because the yields obtained using the same cells cultured at 32 °C and 5% CO_2 were significantly higher and reached 40 mg/L in the best trial among three independent attempts (Supplementary Table S1). Finally, RBD was stored at 4 °C in plastic tubes.

92.5% of the purified RBD expressed at 32 °C eluted from RP-HPLC as a single peak at 21.6 min (Supplementary Fig. S2), while the profile of the protein expressed at 37 °C showed contaminants with MW > 100 kDa, as already observed in gel filtration chromatography (Fig. 1B). Tandem Mass Spectrometry (MS/MS) confirmed RBD identity enabling the recognition of the sequence (Supplementary Fig. S3).

RBD binding activity to the immobilized CR3022-AbSIP was demonstrated by BLI (Supplementary Fig. S4) and flow cytometer analysis showed the binding to the ACE2 receptor (Fig. 2B). Data acquired using FACSCalibur (BD Bioscience) clearly showed the binding-dependent signal shift with respect to the controls (Fig. 2B). A final ELISA assay was useful to detect specific anti-RBD IgG antibodies in human sera of 5 patients recovering from COVID-19 (Fig. 2C).

4. Conclusions

This study describes a reliable protocol suitable for standard laboratory equipment [30] to obtain monomeric and functional SARS-CoV-2 Spike RBD protein at yields of 40 mg/L. Supplementary Table S2 summarizes material and time necessary for RBD production. The major protocol novelties are the use of ExpiCHO transient expression system for RBD production, the expression conditions with temperature set to 32 °C and CO_2 set to 5% and the BLI-dependent monitoring of protein accumulation in the culture media to establish the optimal recovery time. We expect that this approach might be easily applied for producing RBDs of SARS-CoV-2 mutants.

Author statement

All authors (Matteo De March, Michela Terdoslavich, Sulena Polez, Corrado Guarnaccia, Monica Poggianella, Alessandro Marcello, Nataša Skoko and Ario de Marco) of this submitted manuscript confirm that this manuscript has not been published and is not under consideration by any another journal.

Data availability statement

All data supporting this study can be requested to the corresponding authors.

Declaration of competing interest

The authors have declared no conflict of interest.

Acknowledgements

The work was supported by the CRP Collaborative Research Grant SVN20-01 to AdM and by the ICGEB intramural funds to NS. Support from SNAM SpA and Generali SpA to AM is acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2022.106071.

References

[1] S.C. Wu, Progress and concept for COVID-19 vaccine development, Biotechnol. J. 15 (6) (2020), e2000147, https://doi.org/10.1002/biot.202000147.
[2] J.K. Millet, G.R. Whittaker, Host cell proteases: critical determinants of coronavirus tropism and pathogenesis, Virus Res. 202 (2015) 120–134, https://doi.org/10.1016/j.virusres.2014.11.021.
[3] M. Hoffmann, H. Kleine-Weber, S. Schroeder, N. Krüger, T. Herrler, et al., SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor, Cell 181 (2) (2020) 271–280, https://doi.org/10.1016/j.cell.2020.02.052.
[4] Q. Wang, Y. Zhang, L. Wu, S. Niu, C. Song, et al., Structural and functional basis of SARS-CoV-2 entry by using human ACE2, Cell 181 (4) (2020) 894–904, https://doi.org/10.1016/j.cell.2020.03.045.
[5] R. Yan, Y. Zhang, Y. Li, L. Xia, Y. Guo, Q. Zhou, Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2, Science 367 (6485) (2020) 1444–1448, https://doi.org/10.1126/science.abb2762.
[6] L. Vangelista, M. Secchi, Prepare for the future: dissecting the spike to seek broadly neutralizing antibodies and universal vaccine for pandemic coronaviruses, Front. Mol. Biosci. 7 (2020) 226, https://doi.org/10.3389/fmolb.2020.00226.
[7] F. Levi-Schaffer, A. de Marco, Coronavirus disease 2019 and the revival of passive immunization: antibody therapy for inhibiting severe acute respiratory syndrome coronavirus 2 and preventing host cell infection: IUPHAR review, J. Pharmacol. 178 (17) (2021) 3359–3372, https://doi.org/10.1111/bph.15359.
[8] Y. Lou, W. Zhao, H. Wei, M. Chu, R. Chao, M. Liu, Cross-neutralization of RBD mutant strains of SARS-CoV-2 by convalescent patient derived antibodies, Biotechnol. J. 16 (11) (2021), e2100207, https://doi.org/10.1002/biot.202100207.
[9] T.K. Tan, P. Rijal, R. Rahikainen, A.H. Keeble, L. Schimanski, et al., A COVID-19 vaccine candidate using SpyCatcher multimerization of the SARS-CoV-2 spike protein receptor-binding domain induces potent neutralising antibody responses, Nat. Commun. 12 (1) (2021) 542, https://doi.org/10.1038/s41467-020-20654-7.
[10] M.V. Sinegubova, N.A. Orlova, S.V. Kovnir, L.K. Dayanova, P. Vorobiev, High-level expression of the monomeric SARS-CoV-2 S protein RBD 320-537 in stably transfected CHO cells by the EEF1A1-based plasmid vector, PLoS One 16 (2) (2021), e0242890, https://doi.org/10.1371/journal.pone.0242890.
[11] M.A. Ozma, P. Maroufi, E. Khodadadi, Ş. Köse, I. Esposito, et al., Clinical manifestation, diagnosis, prevention and control of SARS-CoV-2 (COVID-19) during the outbreak period, Inf. Med. 28 (2) (2020) 153–165.
[12] M.P. Raphael, J.A. Christodoulides, J.M. Byers, G.P. Anderson, J.L. Liu, et al., Optimizing nanoplasmonic biosensor sensitivity with orientated single domain antibodies, Plasmonics 10 (6) (2015) 1649–1655, https://doi.org/10.1007/s11468-015-9969-3.
[13] S. Oloketuyi, E. Mazzega, J. Zavašnik, K. Pungjunun, K. Kalcher, et al., Electrochemical immunosensor functionalized with nanobodies for the detection of the toxic microalgae Alexandrium minutum using glassy carbon electrode modified with gold nanoparticles, Biosens. Bioelectron. 154 (2020) 112052, https://doi.org/10.1016/j.bios.2020.112052.
[14] C. Tiede, A.A. Tang, S.E. Deacon, U. Mandal, J.E. Nettleship, et al., Adhiron: a stable and versatile peptide display scaffold for molecular recognition applications, Protein Eng. Des. Sel. 27 (5) (2014) 145–155, https://doi.org/10.1093/protein/gzu007.
[15] Y. He, J. Qi, L. Xiao, L. Shen, W. Yu, T. Hu, Purification and characterization of the receptor-binding domain of SARS-CoV-2 spike protein from Escherichia coli, Eng. Life Sci. 21 (6) (2021) 453–460, https://doi.org/10.1002/elsc.202000106.
[16] G.A. Fitzgerald, A. Komarov, A. Kaznadzey, I. Mazo, M.L. Kireeva, Expression of SARS-CoV-2 surface glycoprotein fragment 319-640 in E. coli, and its refolding and purification, Protein Expr. Purif. 183 (2021) 105861, https://doi.org/10.1016/j.pep.2021.105861.
[17] M.L. Bellone, A. Puglisi, F. Dal Piaz, A. Hochkoeppler, Production in Escherichia coli of recombinant COVID-19 spike protein fragments fused to CRM197, Biochem. Biophys. Res. Commun. 558 (2021) 79–85, https://doi.org/10.1016/j.bbrc.2021.04.056.
[18] X. Gao, S. Peng, S. Mei, K. Liang, M.S.I. Khan, et al., Expression and functional identification of recombinant SARS-CoV-2 receptor binding domain (RBD) from E. coli system, Prep. Biochem. Biotechnol. 30 (2021) 1–7, https://doi.org/10.1080/10826068.2021.1941106.
[19] K.L. Tee, P.J. Jackson, J.M. Scarrott, S.R.P. Jaffe, A.O. Johnson, et al., Purification of recombinant SARS-CoV-2 spike, its receptor binding domain, and CR3022 mAb for serological assay, bioRxiv (2020), https://doi.org/10.1101/2020.07.31.231282, 07.31.231282.
[20] O. Farnós, A. Venereo-Sánchez, X. Xu, C. Chan, S. Dash, et al., Rapid high-yield production of functional SARS-CoV-2 receptor binding domain by viral and non-viral transient expression for pre-clinical evaluation, Vaccines 8 (4) (2020) 654, https://doi.org/10.3390/vaccines8040654.
[21] T. Li, H. Cai, H. Yao, B. Zhou, N. Zhang, et al., A synthetic nanobody targeting RBD protects hamsters from SARS-CoV-2 infection, Nat. Commun. 12 (1) (2021) 4635, https://doi.org/10.1038/s41467-021-24905-z.
[22] W.H. Chen, S.M. Chag, M.V. Poongavanam, A.B. Biter, E.A. Ewere, et al., Optimization of the production process and characterization of the yeast-expressed SARS-CoV recombinant receptor-binding domain (RBD219-N1), a SARS vaccine candidate, J. Pharm. Sci. 106 (8) (2017) 1961–1970, https://doi.org/10.1016/j.xphs.2017.04.037.
[23] I. Bagdonaite, A.J. Thompson, X. Wang, M. Søgaard, C. Fougeroux, et al., Site-specific O-glycosylation analysis of SARS-CoV-2 spike protein produced in insect and human cells, Viruses 13 (4) (2021) 551, https://doi.org/10.3390/v13040551.

[24] M. Stuible, C. Gervais, S. Lord-Dufour, S. Perret, D. L'Abbé, et al., Rapid, high-yield production of full-length SARS-CoV-2 spike ectodomain by transient gene expression in CHO cells, J. Biotechnol. 326 (2021) 21–27, https://doi.org/10.1016/j.jbiotec.2020.12.005.

[25] N.K. Jain, S. Barkowski-Clark, R. Altman, K. Johnson, F. Sun, et al., A high-density CHO-S transient transfection system: comparison of ExpiCHO and Expi293, Protein Expr. Purif. 134 (2017) 38–46, https://doi.org/10.1016/j.pep.2017.03.018.

[26] F. Amanat, D. Stadlbauer, S. Strohmeier, T.H.O. Nguyen, V. Chromikova, et al., A serological assay to detect SARS-CoV-2 seroconversion in humans, Nat. Med. 26 (7) (2020) 1033–1036, https://doi.org/10.1038/s41591-020-0913-5.

[27] S. Rajasekharan, R. Milan Bonotto, L. Nascimento Alves, Y. Kazungu, M. Poggianella, et al., Inhibitors of protein glycosylation are active against the coronavirus severe acute respiratory syndrome coronavirus SARS-CoV-2, Viruses 13 (5) (2021) 808, https://doi.org/10.3390/v13050808.

[28] D.C. Liebler, L.J. Zimmerman, Targeted quantitation of proteins by mass spectrometry, Biochemistry 52 (22) (2013) 3797–3806, https://doi.org/10.1021/bi400110b.

[29] M. Milani, M. Donalisio, R. Milan Bonotto, E. Schneider, I. Arduino, et al., Combined in silico docking and in vitro antiviral testing for drug repurposing identified lurasidone and elbasvir as SARS-CoV-2 and CoV-OC43 inhibitors, Antivir. Res. 189 (2021) 105055, https://doi.org/10.1016/j.antiviral.2021.105055.

[30] A. de Marco, N. Berrow, M. Lebendiker, M.H. Gracia-Alai, S. Knauer, et al., Quality control of protein reagents for the improvement of research data reproducibility, Nat. Commun. 12 (1) (2021) 2795, https://doi.org/10.1038/s41467-021-23167-z.