Tampere University

Alina Iakubovskaia

PRODUCTION AND CHARACTERIZA-TION OF RECOMBINANT ANTIBODY VARIANTS NEUTRALIZING SARS-COV-2

ABSTRACT

Alina Iakubovskaia: "Production and Characterization of Recombinant Antibody Variants Neutralizing SARS-CoV-2"
Master's thesis
Tampere University
Faculty of Medicine and Health Technology
Master's Degree Programme in Biotechnology and Biomedical Engineering
Supervisors: Arja Pasternack, Olli Ritvos
Examiners: Vesa Hytönen, Arja Pasternack
April 2022

Coronaviruses are ssRNA viruses, some of which can infect human lung epithelium via binding the ACE2 receptor. The outbreak of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) reported at the end of 2019 has forced the biomedical field to shift the focus to intensive studying of the novel virus, its diagnostics and treatment strategies.

This thesis work aimed to produce recombinant IgG, Fab, scFv and scFv-Fc antibodies against receptor binding site (RBD) of SARS-CoV-2 and to perform their characterization regarding their specificity and overall functionality. The antibody constructs are based on two antibodies (REGN10933 and REGN10987) developed by Regeneron Pharmaceuticals, which have demonstrated high efficiency in neutralizing SARS-CoV-2 in animal testing and humans.

The approach for recombinant antibody production includes the expression of antibodies and antibody fragments using mammalian expression vectors comprising single chain fragment variable (scFv) and IRES-mediated tricistronic genes as well as fusions with mFc and hFc regions of IgG. In particular, this approach allows beneficial outcomes regarding protein yields and stability.

The DNA for the antibody constructs was cloned in *E.coli* and expressed in HEK293 cells. Proteins were purified with affinity chromatography techniques. The quality of the product was assessed with SDS-PAGE gel staining and Western blotting. Obtained results have demonstrated satisfactory purity and yields for most proteins as well as the expected formation of covalent bonds between antibody chains (di-, tetramerization). However, some of the antibody variants' expression and functionality failed presumably due to the possible flaw in the sequence alignment.

Analysis of binding properties of the antibody constructs was performed by pseudovirus neutralization assay against Wuhan, Beta, Delta and Omicron SARS-CoV-2 variants. The assay was carried out on ACE2 overexpressing HEK cells, which allowed to assess specific inhibition of viral entry by neutralizing anti-RBD antibodies and antibody fragments.

The results demonstrated that SCF1-mFc and LIH2-mFc appear to be the most efficient and specific recombinant antibody constructs against RBD of certain SARS-CoV-2 variants, able to neutralize pseudovirus in a dose-dependent manner. However, Omicron variant fully resisted the neutralization. The results have also demonstrated that the presence of Fc region significantly improves the stability and neutralizing ability of recombinant antibodies.

The production system of tricistronic, scFv and scFv-Fc constructs as well as their functionality was mainly successful. Most efficient recombinant antibody constructs will be potentially utilized for research and diagnostic purposes, e.g. for immunological assays detecting SARS-CoV-2.

Keywords: coronavirus, SARS-CoV-2, spike protein, RBD, recombinant antibody, DNA cloning, antibody, tricistronic gene, scFv, Fab, IgG, pseudovirus assay.

TIIVISTELMÄ

Alina Iakubovskaia: "SARS-CoV-2:ta neutraloivien rekombinanttivasta-aineiden tuottaminen ja karakterisointi"
Pro Gradu
Tampereen yliopisto
Lääketieteen ja terveysteknologian tiedekunta
Bioteknologian ja biolääketieteen tekniikan maisterikoulutus
Ohjaajat: Arja Pasternack, Olli Ritvos
Tarkastajat: Vesa Hytönen, Arja Pasternack
Huhtikuu 2022

Koronavirukset ovat ssRNA-viruksia, joista osa voi infektoida ihmisen keuhkoepiteeliä sitoutumalla ACE2-reseptoriin. Vakavan akuutin hengitystieoireyhtymän koronaviruksen-2 (SARS-CoV-2) aiheuttama pandemian puhkeaminen vuonna 2019 on saanut biolääketieteen alan siirtämään painopisteen uuden viruksen, sen diagnostiikan ja hoitostrategioiden intensiiviseen tutkimiseen.

Tämän opinnäytetyön tavoitteena oli tuottaa rekombinantteja IgG-, Fab-, scFv- ja scFv-Fc-vasta-aineita SARS-CoV-2:n reseptorin sitoutumiskohtaa (RBD) vastaan ja karakterisoida niitä spesifisyyden ja yleisen toiminnallisuuden osalta. Vasta-aineet perustuvat Regeneron Pharmaceuticalsin kehittämiin kahteen vasta-aineeseen (REGN10933 ja REGN10987), jotka ovat osoittaneet olevansa erittäin tehokkaita SARS-CoV-2:n neutraloinnissa eläinkokeissa ja ihmisillä.

Tässä työssä rekombinanttivasta-aineita ja niiden fragmentteja tuotettiin nisäkkäiden ekspressiovektoreilla, jotka sisältävät yksiketjuisten vasta-aineiden (scFv) geenejä, IRES-välitteisiä trikistronisia geenejä sekä IgG:n mFc- ja hFc-alueiden fuusioita. Tämä lähestymistapa mahdollistaa hyvät tulokset proteiinien saannon ja stabiilisuuden osalta.

Vasta-ainekonstruktien DNA kloonattiin *E.colissa* ja ekspressoitiin HEK293-soluissa. Proteiinit puhdistettiin affiniteettikromatografiatekniikoilla. Tuotteen laatua arvioitiin SDS-PAGE-geeliväri-jäyksellä ja Western blot -analyysillä. Tulokset osoittivat proteiinien tyydyttävän puhtauden ja saannon sekä odotetun kovalenttisten sidosten muodostumisen vasta-aineketjujen välille (di-, tetramerisaatio). Jotkin vasta-ainekonstruktit eivät kuitenkaan ekspressoituneet tai toimineet toivotulla tavalla, mikä johtui oletettavasti konstruktien suunnittelusta.

Vasta-ainekonstruktien sitoutumisominaisuudet analysoitiin pseudovirusneutralisaatiomääri-tyksellä Wuhan-, Beta-, Delta- ja Omicron-SARS-CoV-2 -variantteja vastaan. Koe suoritettiin ACE2:ta yliekspressoivilla HEK-soluilla, joiden avulla pystyttiin arvioimaan viruksen sisäänpääsyn estämistä neutraloivilla anti-RBD-vasta-aineilla ja vasta-ainefragmenteilla.

Tulokset osoittivat, että SCF1-mFc ja LIH2-mFc näyttävät olevan tehokkaimpia ja spesifisim-piä rekombinanttivasta-ainekonstruktioita tiettyjen SARS-CoV-2-varianttien RBD:tä vastaan, sillä ne pystyvät neutraloimaan pseudoviruksen annosvasteisesti. Omicron-variantin neutralointi ei kuitenkaan onnistunut. Tulokset osoittivat myös, että Fc-alueen lisäys konstruktiin parantaa merkittävästi rekombinanttivasta-aineiden stabiilisuutta ja neutralointikykyä.

Trikistronisten, scFv- ja scFv-Fc-konstruktien tuotantosysteemi sekä niiden toiminnallisuus onnistuivat pääosin hyvin. Tehokkaimpia rekombinanttivasta-ainekonstrukteja voidaan mahdollisesti hyödyntää tutkimus- ja diagnostiikkatarkoituksiin, kuten SARS-CoV-2:n osoittamiseen tarkoitetuissa immunologisissa määrityksissä.

Avainsanat: koronavirus, SARS-CoV-2, spike-proteiini, RBD, rekombinanttivasta-aine, DNA-kloonaus, vasta-aine, trikistroninen geeni, scFv, Fab, IgG, pseudovirusmääritys.

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck -ohjelmalla.

PREFACE

This thesis work was performed in the research group of MD PhD Olli Ritvos under the Department of Physiology of the University of Helsinki.

First of all, I would like to express my gratitude to MD PhD Olli Ritvos and PhD Arja Pasternack for giving me the opportunity to do my master's thesis in their group on such a hot topic these days as SARS-CoV-2. Thanks to your guidance and trust I have gained a ton of valuable knowledge and practical experience in biotechnology and the research field. I would also like to thank medical student Rauno Naves for his work on pseudovirus assays and for teaching me how to perform them.

Lastly, I want to thank my family and friends for their support.

Helsinki, 27 April 2022

Alina Iakubovskaia

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
ACE2	Angiotensin-Converting Enzyme 2
Ag	Antigen
BSA	Bovine Serum Albumin
BSL-2	Biological Safety Level 2
cDNA	Complementary DNA
CHO	Chinese Hamster Ovary cells
CMV	Cytomegalovirus
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DSB	Double-Strand Break
DTT	Dithiothreitol
EMA	European Medicines Agency
ER	Endoplasmic Reticulum
Fab	Fragment antigen-binding
Fc	Fragment crystallizable region
FDA	Food and Drug Administration
HC	Heavy Chain
HCoV	Human Coronavirus
HEK	Human Embryonic Kidney
hFc	Human Fragment crystallizable region
His-tags	Polyhistidine-tags
HRP	Horseradish Peroxidase
IFN	Interferon
IgG	Immunoglobulin G
IMAC	Immobilized Metal Affinity Chromatography
IRES	Internal Ribosome Entry Site
ITR	Inverted Terminal Repeat
kDa	Kilodalton
LB	Lysogeny Broth
LC	Light Chain
LSB	Laemmli Sample Buffer
mAb	Monoclonal Antibody
MERS-CoV	Middle East respiratory syndrome coronavirus
mFc	Murine Fragment crystallizable region
mRNA	Messenger RNA
MTX	Methotrexate
NHEJ	Non-Homologous End Joining
ORF	Open Reading Frame
PAC	Puromycin-N-Acetyltransferase
PAMP	Pathogen-Associated Molecular Pattern
PB	PiggyBac
PBMC	Peripheral Blood Mononuclear Cell

PBS	Phosphate-Buffered Saline
PTM	Post-Translational Modification
RBD	Receptor-Binding Domain
RBM	Receptor-Binding Motif
RE	Restriction Enzyme
RNA	Ribonucleic Acid
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
scFv	Single chain variable Fragment
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SP	Sleeping Beauty
ssRNA	Single-stranded Ribonucleic Acid
TBST	Tris-Buffered Saline and Tween 20
TMPRSS2	Transmembrane Serine Protease 2
VH	Heavy Chain Variable Region
VL	Light Chain Variable Region
VLP	Virus-Like Particle
VOC	Variants of Concern
WHO	World Health Organization

1 INTRODUCTION

The outbreak caused by a novel coronavirus was reported to the World Health Organization (WHO) at the end of 2019 and was later defined as the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) (World Health Organization, 2020). The coronavirus disease 2019 (COVID-19) pandemic has caused a significant load on healthcare as well as boosted the research towards the development of an efficient biomedical tool for diagnostics and treatment of the disease.

Antibodies with high specificity and affinity are widely used in diagnostics and therapeutics for various targets. Production of recombinant antibodies and antibody fragments is a significant field of the pharmaceutical industry and is a constantly developing field in the biomedical research (Ma, 2020). Among the biologics that have been used in COVID-19 therapy, REGN10933 and REGN10987 antibodies generated by Regeneron Pharmaceuticals represent two monoclonal antibodies that bind SARS-CoV-2 spike protein in a non-overlapping manner and neutralize the virus (Baum, 2020a; Weinreich, 2021a). This thesis work aims to produce recombinant antibodies and antibody fragments based on the sequences of REGN10933 and REGN10987, and evaluate their functionality and the success of applied production approaches.

This work provides an overview of SARS-CoV-2, its structure and cell entry mechanism. The use and production process of recombinant antibodies and antibody fragments are also described considering the aspects and elements important for efficient protein expression. The produced antibody constructs are described in detail along with utilized methods and their principles. The results overview the obtained protein yields, quality and neutralization properties of the antibodies, their features and characterization.

2 LITERATURE REVIEW

2.1 Human coronaviruses

Coronaviruses (CoVs) represent a diverse *Coronaviridae* family of enveloped, single-stranded positive-sense RNA viruses. The *Orthocoronavirinae* subfamily consists of four genera: alpha, beta, delta, and gamma. Among them, α-coronavirus and β-coronavirus have become major human pathogens (Table 1) (Zhu, 2020b).

Table 1. Human coronaviruses (Santos-Sánchez, 2020).

Subfamily	Genus	Species
Orthocoronavirinae	Alphacoronavirus	HCoV-229E
		HCoV-NL63
	Betacoronavirus	HCoV-OC43
		HCoV-HKU1
		SARS-CoV
		MERS-CoV
		SARS-CoV-2
	Deltacoronavirus	
	Gammacoronavirus	

There are 7 coronaviruses known until now to infect humans and cause the disease. Human coronaviruses (HCoV) such as HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1 are commonly encountered, causing mild and not long-lasting illness - a common cold. However, people with immunodeficiencies, infants and older adults can develop more serious lower-respiratory tract infections (Chen, 2020).

Other human coronaviruses, the Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV), cause more severe infection and symptoms, such as fever, shortness of breath (dyspnea) often progressing to pneumonia. SARS-CoV and MERS-CoV last emerged at the beginning of the 2000s and 2010s, respectively, causing relatively high mortality (Chen, 2020). In 2019, severe acute respiratory syndrome coronavirus (SARS-CoV-2), which is characterized as a highly infectious and pathogenic virus associated with high mortality, has caused a worldwide pandemic (Schoeman, 2021).

2.2 SARS-CoV-2

In December 2019, a highly infectious novel betacoronavirus, severe acute respiratory syndrome coronavirus (SARS-CoV-2), was first reported and subsequently has spread worldwide. It is known to originate from a cluster of patients with pneumonia in Wuhan, China (Zhu, 2020a). Coronavirus disease (COVID-19) caused by SARS-CoV-2 is a worldwide pandemic, which has a strong impact on society, healthcare, research, economics and other fields.

The virus is mainly spread by respiratory aerosol droplets being prone to infect the upper as well as lower respiratory tracts. The virus enters the cell via an interaction with the host's ACE2 receptors (Zhu, 2020b). The COVID-19 caused by different variants of SARS-CoV-2 may include various symptoms, such as fever, dyspnea, dry cough, chest pain, muscle pain, etc. The manifestations of SARS-CoV-2 can range from the absence of symptoms or mild ones to more severe conditions, such as respiratory failure (Schoeman, 2021).

2.2.1 Viral genome and structure

Novel betacoronavirus SARS-CoV-2 is an enveloped virus with single-stranded, positive-sense RNA (+ssRNA) with a genome of approximately 26–32 kb. The genome contains 14 open reading frames (ORFs) encoding 29 viral proteins (Figure 1) (Yang, 2021).
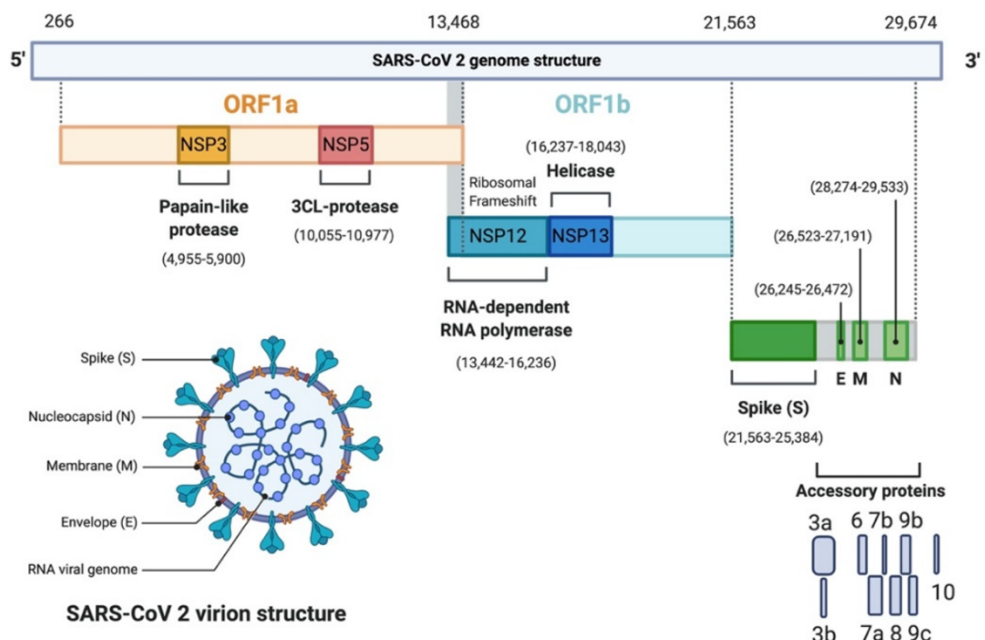

Figure 1. *Genome organization of SARS-CoV-2 and the virion overall structure. The genome encodes two large genes ORF1a (orange) and ORF1b (blue), which encode non-structural proteins. The 3' end genes encode structural and accessory proteins. Figure retrieved from Alanagreh, 2020.*

The 5' end of the genome encodes two overlapping polypeptides, which result in 16 non-structural proteins upon digestion by viral proteases. These proteins are particularly important for viral replication and transcription. The 3' terminus (one-third of the genome) contains four ORFs, which encode all necessary viral structural proteins: the nucleocapsid (N), spike (S) protein, membrane (M) protein and envelope (E) protein (Figure 1). Structural proteins are necessary for the assembly of virion as well as immune response suppression. A set of ORFs for genes encoding accessory proteins essential for regulating the viral infection lies between the structural genes (Yang, 2021).

According to Lu et al. (2020), SARS-CoV-2 has approximately 79% genomic similarity with SARS-CoV and only about 50% with MERS-CoV. All these hCoVs possess the typical structure of betacoronaviruses. As previously mentioned, SARS-CoV-2 has four essential structural N, S, M and E proteins, where the S protein has the key role in viral entry into the host cell. Spike protein is a large protein (approx. 150 kDa) forming a homotrimer - spike complexes on the virion surface (Schoeman, 2021). S glycoprotein enables viral entry via the attachment of the viral S trimer to the host cell surface angiotensin-converting enzyme 2 (ACE2) receptor. This binding causes the conformational changes of the S proteins followed by fusion of the viral and host cell membranes (Zhu, 2020b).

The S protein of SARS-CoV-2 (approx. 1200 residues) is a heavily glycosylated protein, which can be structurally divided into S1 and S2 subunits (Yang, 2021). The S1 subunit is known to be primarily responsible for receptor-binding via its receptor-binding domain (RBD). The RBD, in turn, consists of two subdomains, a receptor-binding motif (RBM) and a core structure, and facilitates the recognition of the host ACE2 receptor. The S2 subunit is known to mediate membrane fusion between the virus and host membrane, inducing the viral genome penetration into host cells (Wu, 2022). Structural representation and S-ACE2 receptor interaction are found in Figure 2 in the next section.

Other essential structural proteins of SARS-CoV-2 are the nucleocapsid (N) proteins, which are multifunctional proteins mainly expressed within infected cells, playing key roles in virus replication, transcription and translation. N is involved in the packaging of the RNA genome and virions as well as the interaction with many host proteins. N is also assumed to interfere in the cell cycle and facilitate inhibition of interferon (IFN) expression, which is assumed to play a role in the viral pathogenicity (Schoeman, 2021).

2.2.2 Cellular entry

The cellular entry of SARS-CoV-2 is facilitated by RBD-ACE2 receptor specific recognition and subsequent interaction between S protein and ACE2 receptor, which leads to proteolytic cleavages and conformational changes required for membrane fusion and release of the viral genome. S protein contains a furin cleavage site between S1 and S2, which is proteolytically cleaved upon binding the host cell to mediate membrane fusion (Figure 2). Another important protease in this context is transmembrane serine protease 2 (TMPRSS2), which cleaves S protein at the S2′ site (Figure 3) enhancing membrane fusion activity of S (Bestle, 2020).
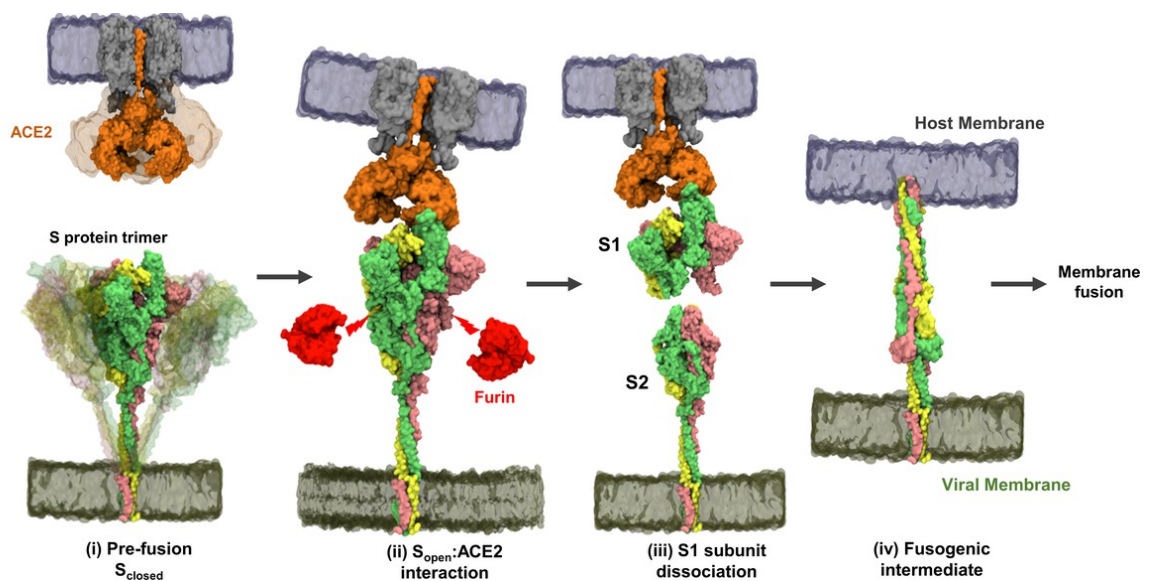

Figure 2. *SARS-CoV-2 host entry steps initiated by recognition and binding to ACE2 receptor mediated by S protein. Figure retrieved from Raghuvamsi, 2021.*

The RBD within S1 subunit is assumed to adopt 'up' or 'down' conformations, where 'up' represents a high-affinity state for the ACE2 receptor. A 'down' state of RBD is a shielded state from receptor binding (Figure 3). ACE2 binding leads to conformational changes promoting proteolysis at the S1/S2 cleavage site (furin), causing dissociation of S1 and S2 subunits (Figure 2). Subsequent conformational changes in separated S2 subunit lead to the formation of an extended fusogenic intermediate structure. This, in turn, promotes the fusion with the membrane of a host cell and the viral entry (Raghuvamsi, 2021).
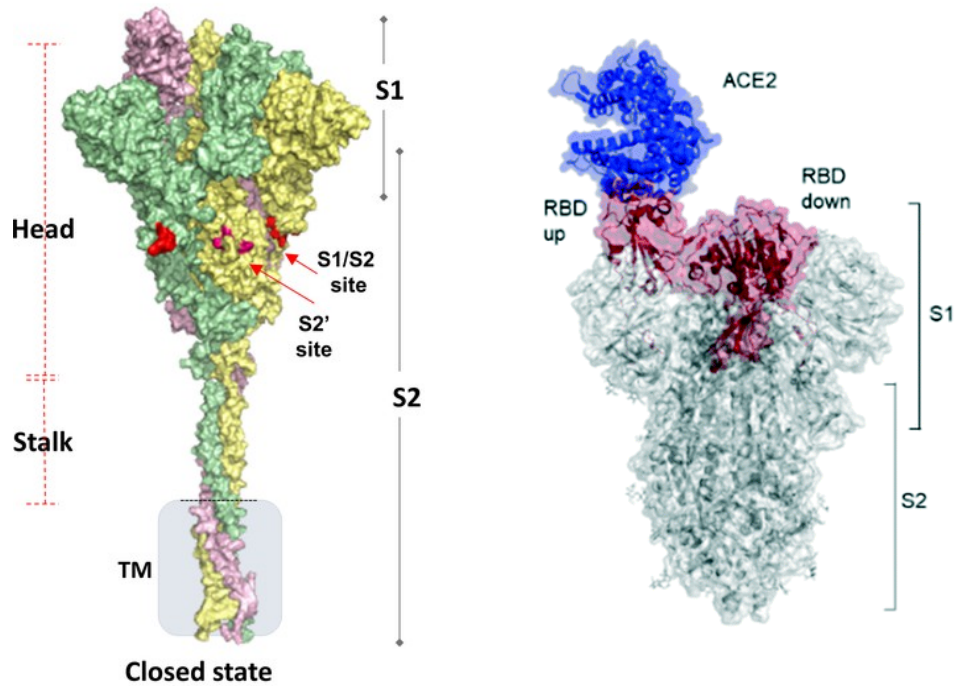
Figure 3. Left: Structure and domain organization of trimeric SARS-CoV-2 spike (S) protein (monomers in yellow, green and pink). Right: Trimeric S protein and its interaction with human ACE2 (blue) via one RBD (red) in 'up' conformation, the other two RBDs (red) are in 'down' conformation. Figures retrieved from Raghuvamsi, 2021 and Pomplun, 2021, respectively.

2.2.3 SARS-CoV-2 variants

Since the beginning of the SARS-CoV-2 pandemic, new variants of the virus have been emerging, affecting viral transmissibility, immune evasion and COVID-19 severity (Mendiola-Pastrana, 2022). The most reported SARS-CoV-2 variants presented changes in S and N proteins. In particular, the RBD region responsible for binding the host's ACE2 receptor is prone to accumulate mutations in SARS-CoV-2. These mutations determine the pathogenicity and virulence of the viral variants, where even a single base change can provide the virus selective advantages and significantly affect its spread (Mendiola-Pastrana, 2022).

There are different nomenclatures used to classify SARS-CoV-2 variants. The letters from the Greek alphabet have been commonly applied for general use. Pango classification referring to genetic lineages is also one of the widely used (Parra-Lucares, 2022). Table 2 represents a brief overview of previously circulating SARS-CoV-2 variants of concern (VOCs) (Alpha, Beta, Gamma) and currently circulating VOCs (Delta, Omicron) that have clinical relevance as of April 2022.

Table 2. *General characteristics of SARS-CoV-2 variants of concern (VOCs) that have been designated by WHO as of April 2022 (World Health Organization, 2022; Parra-Lucares, 2022).*

WHO Label	Pango Lineage	Origin	VOC Designation	Transmissibility	Clinical Response to Anti-SARS-CoV-2 Vaccines
Alpha	B.1.1.7	United Kingdom	December 2020	Higher than previous variants	Vaccines prevent infection
Beta	B.1.351	South Africa	December 2020	Higher than previous variants	Vaccines less effective
Gamma	P.1	Brazil	January 2021	Higher than previous variants	Vaccines less effective
Delta	B.1.617.2	India	May 2021	Significantly higher than previous variants	Efficacy of vaccines lowers
Omicron	B.1.1.529 (BA.1, BA.2 sublineages)	South Africa, multiple other countries	November 2021	Extremely higher than previous variants	Lower efficacy compared to other VOCs

2.2.4 Antibody response to SARS-CoV-2 infections

COVID-19 is characterized by highly variable clinical manifestations. The clinical picture of the disease ranges from asymptomatic to mild and moderate manifestations, which can further progress to respiratory failure as well as multiple other organ disorders in some patients, potentially leading to death (Siracusano, 2021).

Pathogenesis of SARS-CoV-2 begins after the virus binds to epithelial cells in the upper respiratory tract, allowing its potential progression to the lungs and infection of alveolar epithelial cells (Siracusano, 2021). Due to the rapid replication of SARS-CoV-2 in the host cells, it commonly induces a strong immune response. This response is accompanied by pro-inflammatory cytokine and chemokine responses, recruitment of various immune cells of the innate and adaptive immune response, and production of anti-SARS-CoV-2 antibodies. The response may develop as an exaggerated response state, causing typical for COVID-19 cytokine storm syndrome, which is considered to be one of the main causes of severity and death in certain COVID-19 patients (Zhu, 2020b).

The humoral immune response includes the sequential production of anti-SARS-CoV-2 IgM, IgA and IgG targeting epitopes in spike (S) and nucleocapsid proteins (N). These immunoglobulin isotypes targeting distinct antigens naturally possess different dynamics and neutralizing effects (Figure 4) (Siracusano, 2021). The variable dynamic of an antibody response is associated with different factors, such as patients' age, viral load, disease severity, etc. (Pang, 2021).

In COVID-19, IgM, an immunoglobulin with a pentameric structure (Figure 4), is usually produced first in response to the infection and lasts during the acute phase of the disease. IgM has a lower affinity for antigens; however, it has higher avidity for antigens due to its structure. The presence of more specific IgA and IgG is commonly detected after the symptom onset, several days after IgM. IgA, typically dimeric immunoglobulin (Figure 4), is mostly expressed in mucosal tissue, which is crucial for immunological neutralization of the virus at the site of invasion (Pang, 2021). IgG is the most abundant monomeric antibody isotype with high affinity and neutralizing properties against SARS-CoV-2. The studies demonstrate that IgG levels in patients commonly decrease within several months and can last up to a year (Xiao, 2021; Zervou, 2021).
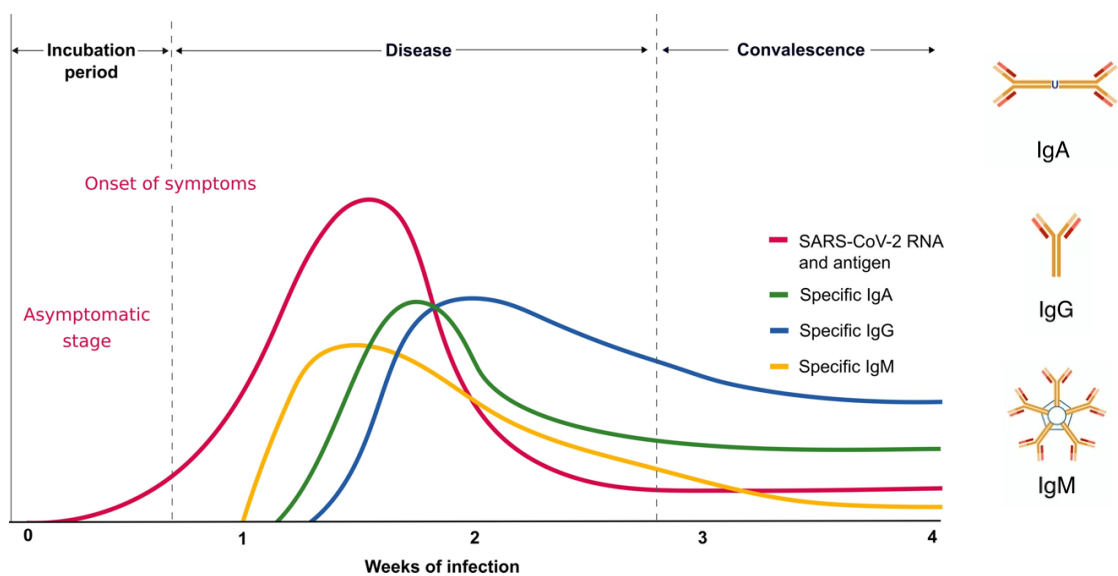

Figure 4. *Schematic humoral response (IgA, IgG, IgM) dynamics of SARS-CoV-2 infection and antibodies' structure. Figure adopted from Azkur, 2020.*

2.2.5 SARS-CoV-2 vaccines

The years of experience in developing vaccines, new approaches and great amounts of resources contributed to the relatively fast development of SARS-CoV-2 vaccines. SARS-CoV-2 vaccines may be divided into four types based on their developmental technology: whole virus vaccines, subunit vaccines, nucleic acid vaccines and viral vector vaccines (Eroglu, 2021).

The traditional approach used for COVID-19 vaccines is the whole virus vaccine, where the virus is either inactivated, e.g. by heat or chemicals, or live attenuated, i.e. weakened. Thus, this type of vaccine utilizes the whole viruses containing all the structural parts enabling proper immune response in a human, but in an inactive form, which makes the virus unable to replicate and cause the disease. The commonly used whole virus inactivated vaccines are, e.g. Chinese BBIBP-CorV (Sinopharm, Beijing) and CoronaVac (Sinovac, Beijing) (Eroglu, 2021).

Another type of vaccine against SARS-CoV-2 is the subunit vaccine. This approach only uses a certain part of the virus to induce an immune response. In particular, S protein and especially its RBD region responsible for ACE2 receptor binding is the key protein subunit used in this technology (Eroglu, 2021). The immunogenicity of the subunits is relatively low and requires various adjuvants to stimulate the immune response. There are several types of subunit vaccines, such as dimeric RBD proteins or virus-like particles (VLPs) mimicking the structure of SARS-CoV-2. Examples of subunit vaccines are, e.g. NVX-CoV2373 (Novavax, US) constructed from the full-length S protein and EpiVacCorona (FBRI, Russia) consisting of three synthesized peptides (short fragments of S protein) conjugated to a carrier protein (Eroglu, 2021; Kyriakidis, 2021).

Nucleic acid COVID-19 vaccines comprise a newer approach of delivering nucleic acid encoding a viral protein to induce an immune response. In this technology, the DNA plasmid or mRNA is delivered via lipid particles into the cells, where the antigen is expressed by a cell itself leading to the immune response to a viral protein (Alshrari, 2022). The mRNA vaccines, such as widely used BNT162b2/Comirnaty (Pfizer/BioNTech, US/Germany), contain mRNA translated into the SARS-CoV-2 S protein in the host cell and have demonstrated a high efficacy (95%) (Polack, 2020). mRNA-1273 (Moderna, US) vaccine applies a similar technology and has been also used worldwide with 94% efficacy (Alshrari, 2022).

Another type of COVID-19 vaccine is the viral vector vaccine, which contains genetically engineered viruses (e.g. retroviruses, lentiviruses, cytomegaloviruses and adenoviruses) to safely deliver genetic material subsequently expressed in a host cell to induce an

immune response. For instance, ChAdOx1-S vaccine (AstraZeneca, UK) contains commonly used adenovirus vectors - harmless carrier viruses containing a gene of SARS-CoV-2 S protein. Although the average efficacy of the vaccine is lower than those produced by Pfizer/BioNTech and Moderna, it possesses similar immunogenicity and is one of the most widely used worldwide. Ad26.COV2.S (Johnson & Johnson, US) is another example of a commonly used adenoviral vector vaccine using the same principle in inducing an immune response (Eroglu, 2021; Alshrari, 2022).

2.3 Recombinant antibodies against SARS-CoV-2

Recombinant antibodies are widely used in diagnostics and treatment of various diseases. Nowadays, infectious diseases, as well as cancer or autoimmune diseases, are the targets for therapeutic antibodies (Roth, 2021). Recombinant antibodies also represent an adjustable diagnostic and research tool, which allows, e.g. to establish the presence of the virus in a sample with high specificity.

In the case of SARS-CoV-2, since the beginning of the pandemic, various monoclonal antibodies have been developed for treatment as well as for diagnostics and research purposes. Numerous biotechnology and pharmaceutical companies have been working on the continuous development of specific antibodies against the constantly emerging SARS-CoV-2 variants to create new tools to diagnose and treat the infection.

The use of recombinant antibodies has been an important treatment option for emergency cases of COVID-19, especially for certain patient groups. The immunotherapy has been particularly beneficial for patients with immunodeficiencies, risk group individuals and elderly people in providing the passive immunity important on the first stages of the infection (Rnjak, 2021). The approach of neutralizing antibody-based immunotherapy utilizes recombinant antibodies specific against the part of the virus. In particular, monoclonal antibodies specific to the RBD of the S protein S1 subunit act as virus-neutralizing molecules, which prevent viral entry and replication subsequently reducing disease severity (Esmaeilzadeh, 2021).

The neutralizing monoclonal antibodies (mAbs) targeting SARS-CoV-2 have been an essential treatment approach for COVID-19. However, high cost, logistical challenges as well as vulnerability to the emergence of new viral variants have affected the prevalence of this treatment option (Robinson, 2022). Nevertheless, some of the developed SARS-CoV-2 neutralizing mAbs have obtained approval from the authorities mostly for emergency use, as they provide immediate passive immunity particularly important in mild-to-moderate COVID-19 cases (Table 3).

Table 3. *Overview of the main neutralizing monoclonal antibodies (mAbs) approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for COVID-19 treatment and their current state (US FDA Administration, 2022; European Medicines Agency (EMA), 2022).*

Neutralizing mAb product	Manufacturer	Target in SARS-CoV-2	Date of First Emergency Use Authorization Issuance by FDA	Current FDA State as of April 2022	Current EMA State as of April 2022
REGEN-COV (casirivimab-imdevimab) (Ronapreve™ in the EU)	Regeneron Pharmaceuticals Inc.	Non-overlapping epitopes on RBD of S protein	21.11.2020	Not authorized due to Omicron prevalence	Authorized for use in the EU
Bamlanivimab-etesevimab	Eli Lilly	Distinct but overlapping epitopes on RBD of S protein	09.02.2021	Not authorized due to Omicron prevalence	-
Xevudy (sotrovimab)	GlaxoSmithKline	Conserved epitope of RBD of S protein	26.05.2021	Not authorized due to Omicron BA.2 sub-variant prevalence	Authorized for use in the EU
Evusheld (tixagevimab-cilgavimab)	AstraZeneca Pharmaceuticals	Non-overlapping epitopes on RBD of S protein	08.12.2021	Authorized for emergency use as pre-exposure prophylaxis for prevention of COVID-19	Authorized for use in the EU
Bebtelovimab	Eli Lilly	Epitope within RBD of S protein	11.02.2022	Authorized for the treatment of mild-to-moderate COVID-19	-

Due to the emergence of Omicron SARS-CoV-2 variant at the end of 2021, the use of bamlanivimab/etesevimab, REGEN-COV (casirivimab/imdevimab) as well as Xevudy (sortovimab) neutralizing mAbs have been later revised and limited by the authorities (FDA), as their neutralizing activity has been demonstrated to be low against this variant (US FDA Administration, 2022).

REGEN-COV casirivimab (REGN10933) and imdevimab (REGN10987) neutralizing monoclonal antibodies developed by Regeneron Pharmaceuticals are of special interest in the context of this paper, as their gene sequences underlie the antibody fragments produced and characterized in this work. REGN10933 and REGN10987 have been considered an effective cocktail as these antibodies bind two different non-overlapping epitopes within the RBD of SARS-CoV-2 spike protein (Figure 5) (Baum, 2020a). These two antibodies administered together have demonstrated reduced viral levels and alleviated symptoms in patients and have been used as a therapeutic antibody cocktail approved by FDA and EMA (Weinreich, 2021b).
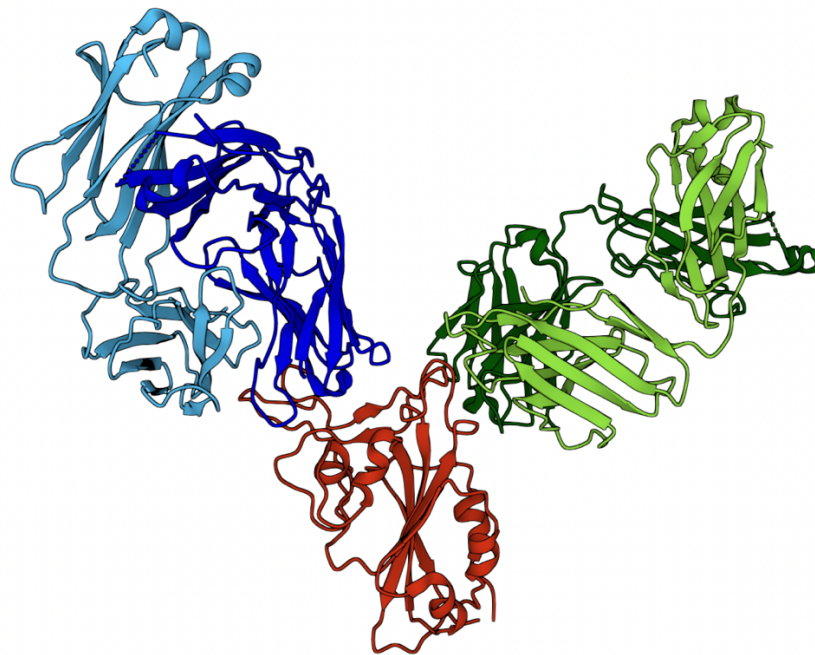

Figure 5. *Cryo-EM image of the complex of REGN10933 (Fab) and REGN10987 (Fab) bound to SARS-CoV-2 RBD. RBD is colored red; REGN10933 heavy and light chains are dark and light blue, respectively; REGN10987 heavy and light chains are dark and light green, respectively. Image from the RCSB PDB (rcsb.org) of PBD ID 6XDG (Hansen, 2020).*

REGEN-COV have been retaining neutralization potency against SARS-CoV-2 muta-tional escape and emerging variants until some point, including e.g., Alpha (UK), Beta (South Africa), Gamma (Brazil), Epsilon (California), Iota (New York) and Kappa (India) variants (Copin, 2021). However, in January 2022, the neutralizing activity of REGEN-COV against the Omicron SARS-CoV-2 variant was stated to be significantly reduced leading to the limitation of its use by FDA (US FDA Administration, 2022).

2.4 Production of monoclonal antibodies

The discovery of the method of producing monoclonal antibodies using hybridoma tech-nology developed by César Milstein and Georges J. F. Köhler in 1975 had an immense impact on the progress of biomedical research, diagnostics and new therapy approaches (Köhler & Milstein, 1975). Over the past decades, the development of recombinant DNA technologies and the creation of combinatorial gene libraries, as well as the emergence of various bioinformatic tools have made the production of recombinant antibodies more advanced. Recombinant technologies make it possible to change the immunochemical properties of mAbs by introducing various modifications into the antibody sequence in order to, e.g. increase the affinity (Alfaleh, 2020).

2.4.1 Structure of antibody and antibody fragments

Immunoglobulins (antibodies) are glycoproteins involved in the immune response in-duced against the pathogen-associated molecular patterns (PAMP) of the pathogen. Im-munoglobulins are also present on the surface of B-lymphocytes, in which they are syn-thesized, in blood, mucous membranes, various body fluids, etc. (Janeway, 2001).

Although only three genetic loci are responsible for antibody synthesis, their immense diversity is provided by mechanisms of DNA rearrangements and the introduction of point mutations (Roth, 2014). This diversity allows the adaptive immune system to generate an antibody response against nearly any antigen. Gene recombination of variable (V), diversity (D) and joining (J) gene segments for the heavy chain and V and J gene seg-ments for the light chain enables an extensive variety of antibodies with diverse specific-ity. The emergence of inaccuracies in V(D)J recombination, as well as mutations, in-creases this diversity providing another level of variety important for the antibody's affinity properties (Roth, 2014).

Immunoglobulin monomers consist of two heavy (HC, ~50–70 kDa) and two light (LC, ~25 kDa) polypeptide chains linked by disulfide bonds (Figure 7). These chains have constant (C) and variable (V) regions. There are five types of heavy chains (α-, γ-, δ-, ε-

, and μ-chains) and two types of light chains (κ-chain and λ-chain) known. According to the type of the heavy chain, 5 classes of immunoglobulins with different properties are distinguished: IgG, IgM, IgA, IgD, IgE (Figure 6). Both heavy and light chains are divided into domains - heavy chains into 4 and light chains into 2. Immunoglobulin structure can be also split into two fragments: Fab (Fragment antigen binding) and Fc (Fragment crystallizable). The flexible hinge region within heavy chains allows the Fab regions to form Y shape with the Fc region (Janeway, 2001).
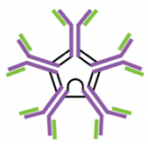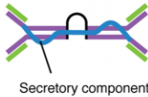
	IgM	IgG	IgA	IgE	IgD
			Secretory component		
Heavy Chain	μ(mu)	γ(gamma)	α(alpha)	ε(epsilon)	δ(delta)
MW	900kDa	150kDa	385kDa	200kDa	280kDa
Percentage of total antibody in serum	6 %	80 %	13 %	0 %	1 %
Function	Primary response, fixes complement. Monomer serves as B-cell receptor	Main blood antibody, neutralizes toxins, opsonization	Secreted into mucus, tears, saliva	Antibody of allergy and anti-parasitic activity	B cell receptor

Figure 6. *Immunoglobulin classes and their main properties. Adopted from Muhammed, 2020.*

One of the key regions of an antibody is the antigen-binding Fv fragment of Fab of the immunoglobulin, formed by the variable domains (V) of the H (VH) and L chains (VL), which bind the epitope of the antigen. This hypervariable region has specific complementary sites to the specific antigenic epitopes. The Fc fragment, in turn, interacts with cell membranes, binds complement and also participates in the transport of IgG (Chiu, 2019).

Figure 7. *Structure of IgG and its fragments: F(ab')2 fragment, Fab fragment, and single chain variable fragment (scFv). Figure adopted from Lin, 2021.*

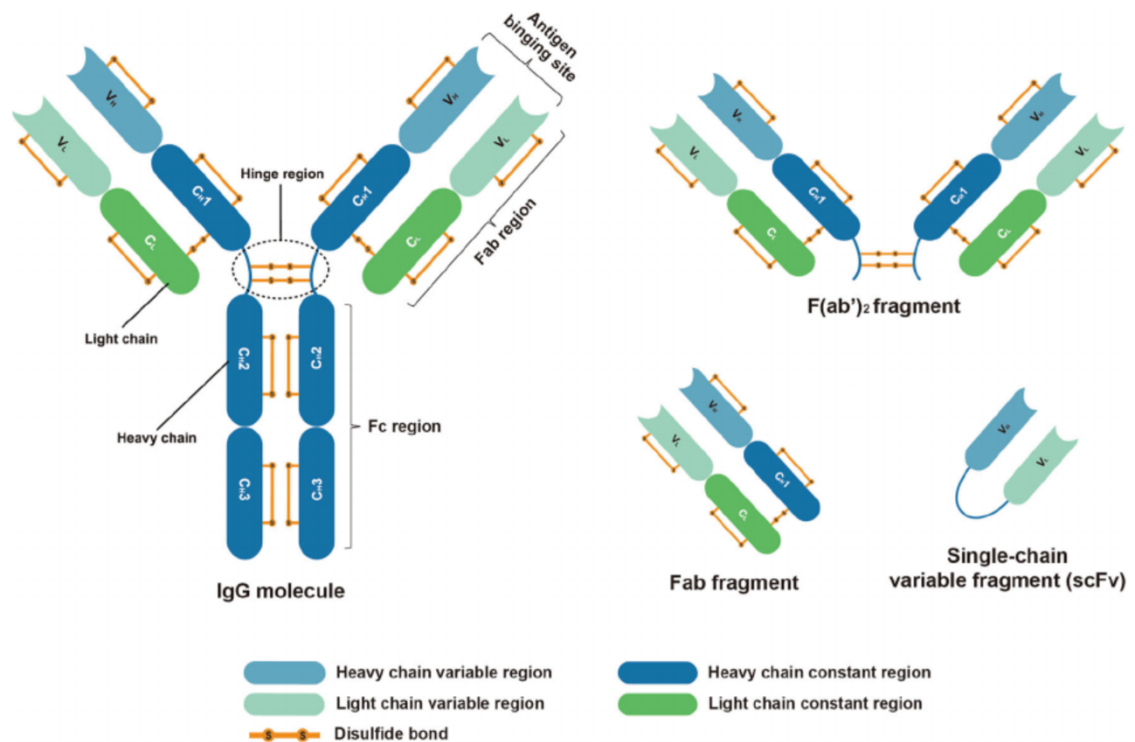Nowadays, recombinant antibody technologies allow to design various antibody fragments, which provide certain advantages over the full-length Abs. There are numerous Ab fragments available with various properties and purposes, e.g. Fab, Fc, as well as single chain variable fragments (scFv), are commonly used in the research and therapeutics (Sifniotis, 2019). Various Ab fragments with key immunological functions are designed and fused for optimization of their molecular size, stability, specificity and immunogenicity. These factors play crucial roles in antibody manufacturing as they address multiple challenges, such as tissue penetration, protein aggregation, yields, stability and functionality (Ahmad, 2012).

Generally, complete mAbs are more stable than the smaller antibody fragments. Glycosylation also plays an important role in protein stability in a whole mAb, while unglycosylated forms are less stable and more prone to aggregation (Ma, 2020). The Fab was the first antibody fragment used in therapeutics and still is the most successful and commonly used mAb format. Fab fragments consist of antibody heavy chain VH and CH1 domains linked to antibody light chain VL and CL domains by a disulfide bond between CL and CH1 (Figure 7). It has a relatively small molecular weight (~ 50 kDa) compared to whole mAb (~ 150 kDa), while possessing similar high specificity and affinity properties. F(ab')$_2$ fragment (~ 110 kDa) is composed of two Fab fragments joined by an IgG

hinge region, which provides this format bivalence and increased avidity (Bates, 2019; Ma, 2020).

scFv is the smallest Ab fragment consisting of the variable heavy (VH) and variable light (VL) domains (Figure 7). These two variable antigen-binding domains linked by a flexible peptide provide high specificity and affinity to the antigen targets. Thus, scFv antibodies possess similar specificity and affinity as full-length Abs, having smaller molecular size (about 30 kDa) and fewer post-translational modifications, which, however, causes less stability than in Fabs (Ma, 2020). Nevertheless, it provides scFv antibodies such advantages as lower production costs, better tissue penetration and access to hidden antigens as well as the ability to be genetically fused to various molecules, such as fluorescent marker proteins for the target detection (Satheeshkumar, 2020).

2.4.2 Hybridoma technology

In 1975, Milstein and Köhler described the first technique developed for stable monoclonal antibody production. The main idea was to obtain a hybrid of a normal antibody-producing cell and a tumor cell. Such a hybrid inherited the ability to synthesize antibodies from a normal cell, and immortality with the ability of unlimited growth from a tumor cell (Köhler & Milstein, 1975).

In this method, mice are injected with an antigen of interest that induces the production of antibodies against it. The spleen of the mice is removed and homogenized to produce a cell suspension. This suspension contains a pool of activated B cells that produce antibodies against the injected antigen (Carvalho, 2017). The spleen cells are then mixed and incubated with myeloma cells, which can grow continuously in culture, and which also lack a salvage nucleotide synthesis pathway. Some of the antibody-producing spleen cells and myeloma cells fuse to form hybrid cells. The cell mixture is placed in a selective medium containing aminopterin, which inhibits nucleotide synthesis and only allows the hybrid cells to grow. Non-malignant myeloma cells as well as B lymphocytes die in such medium (Carvalho, 2017; Mitra, 2021).

The hybrid cells proliferate forming hybridoma clones, which are then screened for production of specific antibodies of interest. Selected hybridomas are eventually cultured to produce large amounts of monoclonal antibodies with high affinity and specificity towards one epitope of a selected antigen (Mitra, 2021).

2.4.3 Phage display

One of the most used approaches of obtaining recombinant antibodies or antibody fragments is the *in vitro* method using a selection from the libraries based on their ability to bind the antigen (Alfaleh, 2020). The group of these methods is called display methods. An important advantage of display methods is the possibility to work simultaneously with the nucleotide and amino acid sequence of each specific antibody variant. Production of recombinant antibody fragments using this approach involves several steps: creation of recombinant antibody DNA libraries, expression and presentation of antibody protein fragments on cell or phage particles and selection of suitable antibody fragments based on their interaction with the antigen. Thus, a full-length antibody specific to a certain antigen can be obtained by first selecting an antibody fragment and then designing the complete nucleotide and amino acid sequence from it (Carvalho, 2017).

The discovery of recombinant antibody fragments, such as Fab and scFv, has contributed to antibody phage display technology development (Alfaleh, 2020). Phage display basically consists of cloning the genes encoding most commonly Fab or scFv antibody fragments amplified from B lymphocytes into bacteriophage plasmid vectors. The genes of antibody fragments library are inserted into the bacteriophage genome into the gene encoding the phage envelope protein. The genes are then transferred to bacteria (e.g., *E. coli*) and infected with an accessory phage, which causes the bacteriophage to assemble, containing an antibody fragment on its surface. The result is a library of bacteriophages, each expressing a certain antibody fragment. The library product is added to the target antigen immobilized on the surface of the tube and then washed. Only bacteriophages containing the gene of antibody fragment with high affinity to the antigen remain on the surface. To select the most high-affinity clones that have bound to the antigen, the phages are removed from the surface and reinfected to *E. coli*. After several rounds of selection, the DNA of the resulting phages is sequenced resulting in the sequence encoding the antibody fragments with the highest affinity (Sidhu, 2001; Alfaleh, 2020).

2.4.4 Single cell sequencing

A developing field of single cell technologies allowed the efficient discovery of mAbs by a rapid screening of mAbs expressed by B cells, especially in the context of their antigen-specificity and functionality (Tiller, 2008). The procedure of discovering the antigen-specific human mAbs starts with the sampling of human blood. The B cells are isolated from peripheral blood mononuclear cells (PBMCs) and enriched. The antigen-specific B cells of interest are then identified and isolated. There are various methods for this purpose,

which are mostly based on flow cytometry and fluorescent staining of cell surface markers. This is followed by sequencing of the Ab encoding genetic material of the cells of interest (Lent, 2021).

The main interest represents the determination of the sequence encoding light chains, heavy chains and particularly their variable regions. This allows to design the recombinant mAbs using the obtained sequence as a template and their subsequent production in different expression systems (Lent, 2021).

2.5 Production of recombinant proteins in mammalian cells

There is a wide range of platforms used for production of recombinant proteins, including prokaryotic and eukaryotic expression systems such as mammalian cells, insect cells, transgenic plants, yeast or bacteria. The choice of a suitable system naturally depends on the protein to be expressed as well as the scale of production – laboratory or large-scale settings (Tripathi, 2019).

Commonly used *E. coli* offers fast growth and high product yields, though lacking proper post-translational modifications (PTMs) and representing a risk of potential endotoxin contamination. Yeasts, such as *S. cerevisiae*, represent well-established hosts for cost-effective commercial production of recombinant proteins, providing necessary PTMs. Transgenic plants have their advantages in good protein folding with PTMs and low over-all cost of production, although the protein purification process is rather complex (Tripathi, 2019). The majority of the recombinant proteins approved for therapeutics are produced in mammalian cell lines, which provide high-quality expression product with humanized glycosylation patterns, although associated with high production costs (Gomes, 2016).

In the case of mAb production, expression in mammalian cells is a more suitable choice due to their ability to express complex proteins of high molecular weight with necessary PTMs, especially glycosylation (Santos, 2018). Nowadays, most utilized expression systems for mAbs commercial production aimed for clinical use are based on Chinese hamster ovary (CHO) cells, murine myeloma cells NS0, murine myeloma cells Sp2/0, human embryonic kidney 293 (HEK 293) cell line and *E. coli* (Sifniotis, 2019).

2.5.1 Large-scale expression

The large-scale production of recombinant proteins is a massive part of the biopharmaceutical industry, including the production of complex biomolecules such as mAbs, enzymes, protein vaccines, hormones, etc. (Baumann, 2016). The speed, yields and cost-effective production are the critical factors in this context. In general, the production process includes optimization of genetic constructs and host cells for expression, large-scale cultivation, processing and purification of recombinant proteins, as well as quality control procedures (Gibaldi, 2013).

The expression systems and overall biopharmaceutical workflow of recombinant protein production mostly depend on the purpose and desired yields of the protein. To meet the requirements and address the challenges of these processes, various high-throughput approaches are applied (Baumann, 2016). Stable gene expression, where the gene is integrated into the host genome, is mostly used for large scale production of recombinant proteins, as it allows greater stability and control of the process. For faster production, frequently needed for the evaluation of protein candidates, a transient gene expression system is preferable as it provides efficient short-term production with the recombinant DNA staying in episomal form (Baldi, 2007).

2.5.2 Design of expression vectors

The design of expression vectors is a crucial stage of recombinant protein production. The development of a genetically engineered construct for expression begins with the selection of the expression system, vector and optimization of the nucleotide sequence encoding the protein. It is necessary to adjust the codon composition for high-level expression. The amino acid composition of the protein is optimized to reduce the probability of, e.g. sedimentation, aggregation and unwanted immunogenicity (Kiyoshi, 2014). In addition, it is important to ensure proper protein folding of chains, considering their interactions and bonding with each other. There are currently programs that allow optimization of the nucleotide sequence of genetically engineered constructs for recombinant protein production *in silico* with the help of computational simulations (Tripathi, 2019).

There are several general genetic elements essential for mammalian expression vectors: a suitable and transcriptionally active promoter; a transcription terminator; translational signals such as optimal translational initiation sequence (the Kozak sequence (Kozak, 1999)) and translation termination codon; prokaryotic origin of replication and selection marker for vector cloning in bacteria; as well as selection markers for stable cell line selection (Makrides, 2003).

Expression vectors are commonly the plasmids - circular DNA molecules encoding both the protein gene itself and the essential accessory elements. Promoter is one of the critical elements considered in an expression vector design, as a selection of an optimal promoter greatly affects the expression levels and stability of a recombinant gene. For instance, the cytomegalovirus (CMV) promoter is frequently used for expression in eukaryotic cells, ensuring high transcription efficiency (Kiyoshi, 2014).

An efficient mammalian expression in terms of protein yields in both transient and stable transfection settings can be achieved with the CMV early enhancer combined with the chicken beta-actin promoter (CAG) (Dou, 2021). CAG promoter consists of the following regulatory elements: (C) CMV early enhancer element; (A) the promoter region, the first exon, and the first intron of chicken beta-actin gene; and (G) the splice acceptor of the rabbit beta-globin gene. The chicken beta-actin contains a CpG island, which helps to prevent the promoter methylation and keep the promoter active for a longer time, compared to CMV promoter (Merck, 2022; Dou, 2021).

The vector must also contain selectable markers, often antibiotic resistance genes, for the cell line selection. This is necessary to select the cells carrying the vector - when the antibiotic for eukaryotic cells is added, cells that do not carry the vector die. In addition, as preliminary manipulations of the vector (e.g., the cloning and subcloning) are commonly performed in bacteria, the expression vector should also include a second antibiotic resistance gene for bacteria (e.g., ampicillin resistance gene) for selection purposes with the same principle (Li, 2010).

In the case of recombinant Ab production, the expression settings of heavy and light chains are considered. Since the antibody includes separate heavy and light polypeptide chains - either a two-plasmid expression system or a single vector containing both genes is used. The latter option is preferable as it allows more precise control of the ratio of light and heavy chains production (Kiyoshi, 2014). It is known that under natural conditions the B cells synthesize light chains in greater amounts than heavy chains, and this ratio of synthesis rates is also the most favorable for antibody production (Schlatter, 2005; Ho, 2013).

The control of the levels of simultaneous gene expression (such as LC and HC) can be achieved by using internal ribosome entry site (IRES) elements to express the genes of interest linked in one transcript under a single promoter. When IRES is placed between the genes in a single vector, the ribosomes can bind IRES in the middle of the transcript, allowing the cap-independent translation of the following gene (Ho, 2013). The use of IRES elements may present a problem, as the first gene is known to be expressed more

efficiently, than the following genes. However, in the case of production of Abs, this presents an advantage as a tool for the control of the optimal level of LC and HC gene expression, which aids in preventing aggregation and increasing conformational stability of recombinant Abs (Ho, 2013; Makrides, 2003).

Another important element for recombinant proteins is a signal peptide, which determines the destination of a synthesized protein. Signal peptides are signal sequences of 15-30 amino acids at the N-terminus suitable to target a specific organelle or for the secretion from the cell. Proteins that function in cytosol lack signal peptides (Makrides, 2003). There are typically three regions in a signal peptide: a positively charged amino-terminal N-region, a central hydrophobic H-region, and a polar carboxy-terminal C-region followed by a signal peptidase cleavage site. For instance, a signal sequence of a protein aimed for endoplasmic reticulum (ER) contains hydrophobic amino acids that promote protein's entry into the lipid bilayer membrane and that is cleaved by a signal peptidase in the ER lumen (Makrides, 2003).

The presence of a fusion moiety for recombinant protein purification should be also considered at the stage of expression vector design. For instance, polyhistidine tag on N- or C-terminus is widely used for affinity purification (Makrides, 2003). For instance, affinity purification is performed utilizing the high affinity of histidine residues for nickel ions. The presence of a purification tag, however, can potentially affect the properties of certain proteins, which can be overcome by including a protease cleavage site and performing corresponding digestion (Debeljak, 2006).

2.5.3 Transfection and transposition

Transfection is the process of introducing purified nucleic acids into a eukaryotic cell by means other than through a virus. There are two main types of transfection: transient and stable transfection (Baldi, 2007). The choice of the transfection strategy depends on the purpose and desired yields of the recombinant protein.

In transient transfection, the DNA is introduced into the cell without integrating it into the host genome and remaining in epichromosomal form. The DNA is gradually removed as the cells proliferate into new cells without copying non-integrated DNA of interest (Gupta, 2016). In this way, the DNA material of interest is not transferred to the new cells and is eventually lost, which naturally decreases the expression levels of these genes over time. This approach allows fast and short-term expression up to 10 days post-transfection without the cell line selection. It is typically used for research purposes and clinical trials period, which do not require high and long-term expression (Baldi, 2007).

In stable transfections, the genes of interest become a part of the cell genome, either by adding to or replacing some of the host DNA. When the cell proliferates, the recombinant genes are transferred to the next descendants. This provides a stable long-term expression with high yields, although it requires a time-consuming cell line selection process (Gupta, 2016).

The mammalian transgenesis for stable recombinant protein expression comprises a wide range of approaches, including the use of various DNA transposon systems. DNA transposons from different organisms have been characterized over the last several decades, such as commonly used Tol2, piggyBac and Sleeping Beauty, which are among the most efficient systems used for DNA integration into the mammalian cell's genome (Wu, 2006).

DNA transposons are mobile DNA sequences that can move and integrate into different locations in the genome. The system requires a transposase enzyme to catalyze this process by cut-and-paste principle. The transposase construct can be co-transfected with transposon-containing plasmid DNA and expressed to integrate the target DNA into the genome (Figure 8). The expression vector should include the transposon-specific inverted terminal repeats (ITRs) sequences on both ends of the transposon vector, containing the DNA fragment of interest in between. The transposase enzyme recognizes and binds ITRs, cuts and transfers the gene into a host cell genome (Tschorn, 2020).
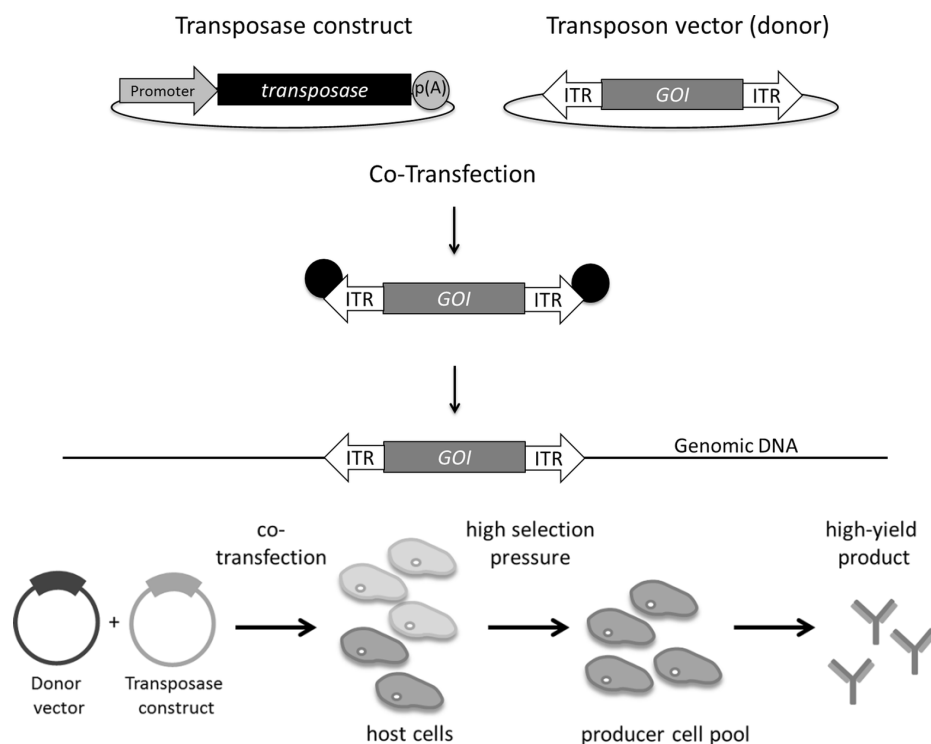

Figure 8. A co-transfection transposon system resulting in integration of the gene of interest (GOI) into the host genome for a stable transfection. Figure adopted from Tschorn, 2020.

During the insertion, transposases open the DNA creating a double-strand break (DSB) and insert the transposon fragment into the locus. The DNA ligation is mediated by DSB repair pathways, involving the non-homologous end joining pathway (NHEJ) (Tschorn, 2020).

PiggyBac (PB) is a highly efficient and widely used transposon isolated from the cabbage looper moth - a mobile genetic element that efficiently transfers between vectors and chromosomes through the described cut-and-paste mechanism. The high activity of the PB transposon system allows the genes of interest between the two ITRs in the PB vector to be easily mobilized into the target genome. The TTAA-specific PB transposon allows the integration over nearly the whole host genome, which makes it a useful transposon system in genetic engineering (Wu, 2006).

Sleeping Beauty (SP) is another frequently used active transposon for mammalian transgenesis. SP represents synthetic sequences derived from transposons found in the white cloud minnow, atlantic salmon and rainbow trout (Tschorn, 2020). Tol2, a transposon originating from medeka fish, is another transposon system alternative, which, however, shows lower transposition efficiencies as compared to SB and PB (Balasubramanian, 2016).

The transfer of the DNA vectors into mammalian cells may be performed by a variety of transfection techniques, including calcium phosphate, electroporation, cationic lipid-based lipofection, and polymer-based methods (Li, 2010). Electroporation and lipofection have been usually the most common choices used for stable transfection. Electroporation is one of the most used transformation methods, where the electrical impulses create transient pores in the cell membrane allowing the DNA to enter the cells. In the lipofection method, the cationic lipids bind to negatively charged plasmid DNA facilitating its delivery into the cell (Jacobsen, 2004).

2.5.4 Cell line selection

Upon successful stable transfection, a selection is performed for the generation of stably transfected cell lines that provide long-term and high-level expression of the protein of interest. Selectable markers are used to screen the cells for stably integrated plasmid DNA, which contains the gene encoding the selectable protein. This allows relatively simple detection of plasmid-containing cells to establish a stable cell line (Li, 2010).

There are various selection systems available for mammalian cells depending on selectable markers. Selectable markers can be categorized into two groups: metabolic selectable markers and antibiotic selectable markers. A metabolic selectable marker is,

e.g. a dihydrofolate reductase (DHFR) gene working along with selectable reagent methotrexate (MTX). MTX inhibits DHFR leading to cell death, which is avoided with the increased DHFR expression, indicating a stable transfection (Kaufman, 2000). Some of the commonly used antibiotic selectable markers are puromycin resistance gene (*pac)* encoding puromycin N-acetyltransferase or aminoglycoside 3'-phosphotransferase gene conferring antibiotic resistance to kanamycin and neomycin for the cells under selection (Li, 2010; US Biological, 2022).

2.5.5 CHO and HEK293 cells

Several different mammalian cells can be used for recombinant protein expression. Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells are among the most commonly used in research and commercial production (Gomes, 2016).

CHO cells represent the predominant mammalian expression system used for therapeutic proteins production (up to 70 % of the market). CHO cells are relatively easy to work with and generally suitable for large-scale production. Due to a humanlike glycosylation pattern, the proteins produced in CHO cells are safe to use in humans without inducing immune reactions. The cost of production, however, is quite high due to the demanding culturing conditions with costly and highly optimized culture media. (Gupta, 2016).

Human embryonic kidney 293 (HEK293) cells are another widely used cell line in protein production and biomedical research. They were obtained in 1973 by the transformation of the kidney cells of an aborted human embryo with sheared Adenovirus 5 DNA. As a result, the adenoviral genome fragment containing E1A and E1B genes was integrated into chromosome 19 of HEK293. This resulted in immortalized human embryonic kidney cells, as E1A and E1B proteins interfere with the cell cycle control and prevent apoptosis (Lin, 2014).

While CHO cells are mostly used in large-scale stable production, HEK293 cell line is commonly preferred for research purposes, due to the ease and speed of transfection and expression of relatively small quantities of recombinant proteins needed for studies. In particular, transient protein expression in HEK293 cells is commonly used for fast recombinant protein production in the context of research and development (Sifniotis, 2019). HEK293 cells provide PTMs essential for recombinant proteins, rapid growth rate, high yields and overall ease of manipulation. HEK293 derived cell lines are routinely used to produce, e.g. recombinant antibodies or viral particles in viral vaccine production (Tan, 2021; Fontana, 2014).

3 OBJECTIVES

This work aims to produce the recombinant IgG, Fab, scFv and scFv-Fc antibodies against RBD of SARS-CoV-2. The production includes 10 different antibody constructs, which are based on the synthetic cDNA sequence of two antibodies developed by American biotechnology company Regeneron Pharmaceuticals: REGN10933 and REGN10987 (Regeneron Pharmaceuticals, 2022). Casirivimab (REGN10933) and imdevimab (REGN10987) have been an effective therapeutic mAb combination when used together, as these antibodies bind two non-overlapping epitopes within the RBD of the SARS-CoV-2 spike protein. Casirivimab/imdevimab has shown high efficiency in neutralizing SARS-CoV-2 in experimental animals as well as reducing viral levels and alleviating the symptoms in humans (Baum, 2020a; Weinreich, 2021a).

The general novelty of this thesis work lies in the approach of designing and producing antibodies. In particular, the approach includes the production of antibodies' functional fragments, as distinct from the original one used by Regeneron Pharmaceuticals. This implies the use of the expression vectors, which express single chain fragment variable (scFv) and IRES-mediated tricistronic LC and HC, that are described in the methods section.

The efficient production of high-quality antibodies is one of the main challenges in the biopharmaceutical industry (Sifniotis, 2019). Previous studies have described the enhanced generation of recombinant antibodies using the mentioned approaches (Ahmad, 2012; Ho, 2012; Yeo, 2018). These methods combined with the efficient properties of anti-SARS-CoV-2 casirivimab and imdevimab antibodies have a potential to serve as a useful tool for research and diagnostics in virology and related fields. One of the main aims of this study is to evaluate the feasibility of these production approaches for the recombinant antibodies, their production rates and functionality.

To summarize, the overall aim of the work is to generate SARS-CoV-2 neutralizing monoclonal recombinant antibodies and antibody fragments and perform the assessment of their binding properties as well as the functionality of the production approach. The detailed goals of this work are:

1. To produce pPB-CAG expression vectors from synthetic cDNAs by DNA subcloning and cloning in *E.coli*

2. To transfect and perform a selection of HEK293F cell line

3. To express recombinant antibody constructs and purify them with the appropriate protein purification method

4. To perform SDS-PAGE and Western blot analysis to estimate the proteins purity, yields and conformation

5. To perform SARS-CoV-2 pseudovirus neutralization assay to assess the antibody variants' neutralization properties against the viral entry into the cells

6. To characterize the antibody constructs and discuss the results

4 MATERIALS AND METHODS

4.1 Antibody constructs

The following antibody variants were produced in this thesis work:

1. pPB-CAG-LIH1-8his

2. pPB-CAG-LIH2-8his

3. pPB-CAG-LIH1-mFc-6his

4. pPB-CAG-LIH2-mFc-6his

5. pPB-CAG-LIH1-hFc

6. pPB-CAG-LIH2-hFc

7. pPB-CAG-SCF1-mFc-6his

8. pPB-CAG-SCF2-mFc-6his

9. pPB-CAG-SCF1-8his

10. pPB-CAG-SCF2-8his

pPB-CAG – PiggyBac vector backbone, CAG promoter; mFc – Fc region of a mouse IgG; hFc – Fc region of human IgG; his – histidine tag. Sequences for LIH1 and SCF1 are REGN10987 antibody derived, and LIH2 and SCF2 are derived from REGN10933 SARS-CoV-2 antibody.

4.1.1 LIH constructs

Based on the sequence from PBD (ID: 6XDG), codon-optimized cDNAs were designed. The LIH constructs comprise the IRES-mediated tricistronic vector, which can express three genes separately under the control of one promoter. In the case of Fab constructs, an IRES sequence was placed so that the heavy chain region was encoded by the same mRNA after the light chain. Another following IRES was placed in the construct coming before the puromycin acetyltransferase gene. This design resulted in the tricistronic constructs for Fab and Fab-Fc (form full IgG). In addition, a signal peptide sequence was placed in front for the secretion of the protein.

The variants without Fc regions (Fabs) include the light chain (LC) and Fab part of heavy chain (VH, CH1) of IgG as well as puromycin-resistance gene (*pac*). The expressed LC

and Fab part of HC (VH, CH1) form a dimeric Fab antibody fragment via disulfide bond between CH1 and CL domains (Figure 9).

LIH1 and LIH2 variants (Fab): 5' Light chain – IRES – Heavy chain (Fab) – IRES – *pac* 3'

The sequence of constructs containing Fc fragments include the light chain (LC), Fab part of heavy chain (VH, CH1) fused with Fc (CH2, CH3), and puromycin-resistance gene (*pac*). Expressed LC and HC with Fc region form a full-length IgG upon tetramerization via disulfide bonds (Figure 9). mFc and hFc sequences are found in Appendices 5-6.

LIH1-Fc and LIH2-Fc variants: 5' Light chain – IRES – Heavy chain (Fab) – Fc – IRES – *pac* 3'

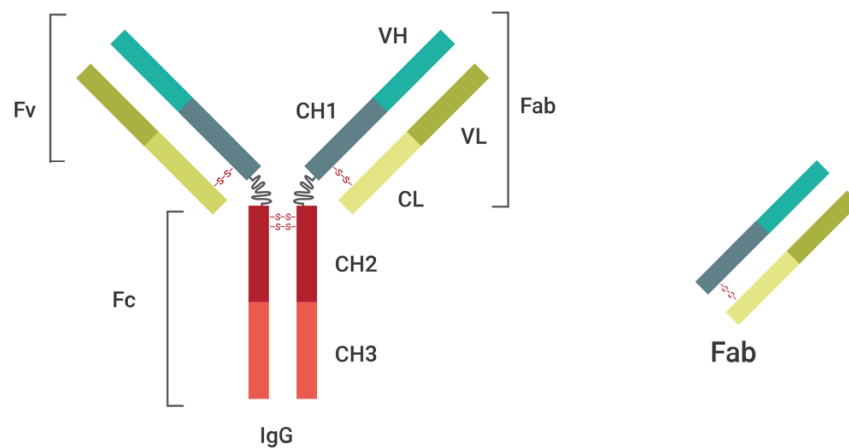**Figure 9.** *Schematic structure to of full-length IgG and Fab fragment. Figures adopted from BioAtla, n.d.*

The DNA sequence of LIH1 (2145 bp) and LIH2 (2133 bp) inserts contain several restriction sites important for cloning and subcloning (Figure 11). The DNA sequences are found in Appendices 1-2. The amino acid sequence contains signal peptide MKWVT-FISLLFLFSSAYS or MPLLLLLPLLWAGALA for protein secretion from the cells.

LIH1 (2145bp) amino acid sequence:

Light chain (234 aa)

```
MKWVTFISLL FLFSSAYSQS ALTQPASVSG SPGQSITISC TGTSSDVGGY
NYVSWYQQHP GKAPKLMIYD VSKRPSGVSN RFSGSKSGNT ASLTISGLQS
EDEADYYCNS LTSISTWVFG GGTKLTVLGQ PKAAPSVTLF PPSSEELQAN
KATLVCLISD FYPGAVTVAW KADSSPVKAG VETTTPSKQS NNKYAASSYL
SLTPEQWKSH RSYSCQVTHE GSTVEKTVAP TECS
```
IRES in between (approx. 600 bp)

Heavy chain (without Fc), **8his** (264 aa)

```
MATTMPLLLL LPLLWAGALA QVQLVESGGG VVQPGRSLRL SCAASGFTFS
NYAMYWVRQA PGKGLEWVAV ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY
LQMNSLRTED TAVYYCASGS DYGDYLLVYW GQGTLVTVSS ASTKGPSVFP
LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHGGS
GGSGGSHHHH HHHH
```

LC and HC (without Fc) should form a dimeric Fab-8his protein
(234 aa + 264 aa = 398 aa) by disulfide bonds.

LIH2 (2133 bp) amino acid sequence:

Light chain (230 aa)

```
MPLLLLLPLL WAGALADIQM TQSPSSLSAS VGDRVTITCQ ASQDITNYLN
WYQQKPGKAP KLLIYAASNL ETGVPSRFSG SGSGTDFTFT ISGLQPEDIA
TYYCQQYDNL PLTFGGGTKV EIKRTVAAPS VFIFPPSDEQ LKSGTASVVC
LLNNFYPREA KVQWKVDNAL QSGNSQESVT EQDSKDSTYS LSSTLTLSKA
DYEKHKVYAC EVTHQGLSSP VTKSFNRGEC
```
IRES in between (approx. 600 bp)

Heavy chain (without Fc), **8his** (264 aa)

```
MATTMPLLLL LPLLWAGALA QVQLVESGGG LVKPGGSLRL SCAASGFTFS
DYYMSWIRQA PGKGLEWVSY ITYSGSTIYY ADSVKGRFTI SRDNAKSSLY
LQMNSLRAED TAVYYCARDR GTTMVPFDYW GQGTLVTVSS ASTKGPSVFP
LAPSSKSTSG GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS
GLYSLSSVVT VPSSSLGTQT YICNVNHKPS NTKVDKKVEP KSCDKTHGGS
GGSGGSHHHH HHHH
```

LC and HC (without Fc) should form a dimeric Fab-8his protein
(230 aa + 264 aa = 394 aa).

4.1.2 SCF constructs

The single chain fragment variable (scFv) fragments consist of the variable part of the
heavy chain (VH) and the variable part of the light chain (VL) connected by a peptide
linker (Figure 10). The scFv versions were made by extracting only the regions from the
sequence encoding the variable regions from the light and heavy chains. These variable

regions (VH and VL) were connected to each other with an extra linker, comprising a single sequence.

This method of antibody fragments production allows the production of rather stable Ab fragments with small molecular weight and higher yields (Ahmad, 2012; Hust, 2007). The mFc domain fused with the constructs provides the dimerization features to the protein, which enhances its binding properties as well as improved its stability.

SCF1 and SCF2 (single chain Fv) variants: 5' Variable light chain – linker – Variable heavy chain – flexible linker

SCF1-mFc and SCF2-mFc variants: 5' Variable light chain – linker – Variable heavy chain – linker – mFc 3'
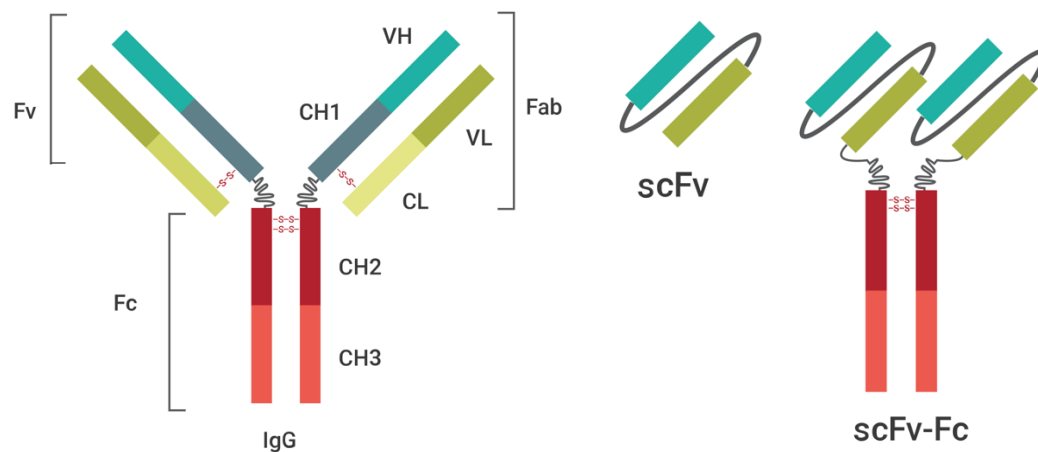
Figure 10. *Schematic structure of full-length IgG, scFv and scFv-Fc antibody fragments. Figures adopted from BioAtla, n.d.*

Similar to LIH inserts, the DNA sequences of SCF1 (976 bp) and SCF2 (961 bp) contain same restriction sites for subcloning (Figure 11). The DNA sequences are found in Appendices 3-4. The amino acid sequence contains signal peptides MKWVTFISLLFLFS-SAYS or MPLLLLLPLLWAGALA, variable domain of light chain (VLC), linker GGGGSGAGGSGGGGTGGGGS, variable domain of heavy chain (VHC), linker GGGGSGGGGSGGGGSGGGGSGGGGS and 8-histidine tag.

SCF1 construct (303 aa): **signal peptide**, VLC, <u>**linker**</u>, VHC, **linker,** 8His

```
MKWVTFISLL FLFSSAYSQS ALTQPASVSG SPGQSITISC TGTSSDVGGY
NYVSWYQQHP GKAPKLMIYD VSKRPSGVSN RFSGSKSGNT ASLTISGLQS
EDEADYYCNS LTSISTWVFG GGTKLTVLGQ GGGGSGAGGS GGGGTGGGGS
QVQLVESGGG VVQPGRSLRL SCAASGFTFS NYAMYWVRQA PGKGLEWVAV
ISYDGSNKYY ADSVKGRFTI SRDNSKNTLY LQMNSLRTED TAVYYCASGS
DYGDYLLVYW GQGTLVTVSS GGGGSGGGGS GGGGSGGGGS GGGGSHHHHH
HHH
```

SCF2 construct (298 aa): **signal peptide**, VLC, <u>**linker**</u>, VHC, **linker,** 8His

```
MPLLLLLPLL WAGALADIQM TQSPSSLSAS VGDRVTITCQ ASQDITNYLN
WYQQKPGKAP KLLIYAASNL ETGVPSRFSG SGSGTDFTFT ISGLQPEDIA
TYYCQQYDNL PLTFGGGTKV EIKRTGGGGS GAGGSGGGGT GGGGSQVQLV
ESGGGLVKPG GSLRLSCAAS GFTFSDYYMS WIRQAPGKGL EWVSYITYSG
STIYYADSVK GRFTISRDNA KSSLYLQMNS LRAEDTAVYY CARDRGTTMV
PFDYWGQGTL VTVSSGGGGS GGGGSGGGGS GGGGSGGGGS HHHHHHHH
```

4.2 DNA cloning

Synthesized inserts LIH1 (2145bp), LIH2 (2133bp), SCF1 (976bp) and SCF2 (961bp) were purchased in pUC57-simple cloning vectors (Gene Universal) (plasmid maps in Appendix 7). DNA cloning and subcloning of the antibody constructs were carried out in competent *E. coli* DH5α cells (Invitrogen) - one of the most common laboratory *E. coli* strains used for optimal and stable amplification of small plasmid DNA. 5µg of pUC57-simple cloning vectors were transformed into *E. coli* DH5α cells using the heat shock (+42°C) method. After the incubation (+37°C, 1h on orbital shaker), transformed bacteria were transferred onto the LB agar plates (A-100) containing 100 µg/ml of ampicillin and incubated at +37°C overnight (o/n). The presence of ampicillin prevented the growth of the bacteria colonies not containing the desired plasmid possessing the ampicillin resistance gene.

On the following day, two individual colonies of each plasmid were collected and further grown in the 5 ml LB solution containing 10 µl of ampicillin (+37°C, o/n on shaker). The replicated plasmid DNA was extracted the following day using GeneJET Plasmid Miniprep Kit (Thermo Scientific™) protocol. The concentration and purity of the extracted pUC57-simple plasmids were measured by NanoDrop™ 2000 Spectrophotometer (Thermo Scientific™).

Test restriction digestion was performed on the obtained plasmid DNA to ensure the correctness of the desired restriction sites for EcoRI-HF and NotI-HF restriction enzymes

(RE) (New England Biolabs Inc.). 12 μl of total restriction mix was ran in 1% agarose gel by gel electrophoresis. Restriction enzyme digestions for further subcloning of the inserts were performed on 5 μg of the DNA with EcoRI-HF and NotI-HF restriction enzymes for pPB-CAG-8His vectors and EcoRI-HF and BamHI-HF RE for pPB-CAG-mFc-6His vectors (Figure 12) in CutSmart™ Buffer (New England Biolabs Inc.). The digested inserts (37°C, o/n) were separated by gel electrophoresis and cut out from the gel with a scalpel. The inserts were extracted from the agarose gel by GeneJET Gel Extraction Kit (Thermo Scientific™). Protocols for the RE-digestions are found in Appendices 8-9.

The inserts were subcloned into pPB-CAG expression vectors suitable for protein expression in mammalian cells. The vectors' backbones were obtained beforehand from other plasmids by cutting out the unwanted inserts by EcoRI-HF and NotI-HF RE for pPB-CAG-8His vectors and by EcoRI-HF and BamHI-HF RE for pPB-CAG-mFc-6His vectors (Figure 12). In case of the construct possessing hFc region, the hFc DNA sequence was subcloned from synthesized pUC57-simple cloning vectors (Gene Universal) as an insert (NotI-HF and BglII RE in NEB2 (New England Biolabs Inc.)). The hFc inserts were ligated into the vectors obtained from the constructs with mFc region, which was cut out by NotI-HF and BamHI-HF RE (Figure 13). BamHI-HF and BglII generate compatible ends, which cannot be cut by either of the REs after ligation. The ligation of the inserts and vectors were performed using T4 DNA Ligase in T4 Ligase Reaction Buffer (New England Biolabs Inc.) overnight at +4°C. The recognition sites of used RE are presented in Figure 11.

5´... G A A T T C ... 3´   5´... G C G G C C G C ... 3´
3´... C T T A A G ... 5´   3´... C G C C G G C G ... 5´

EcoRI-HF   NotI-HF

5´... G G A T C C ... 3´   5´... A G A T C T ... 3´
3´... C C T A G G ... 5´   3´... T C T A G A ... 5´

BamHI-HF   BglII

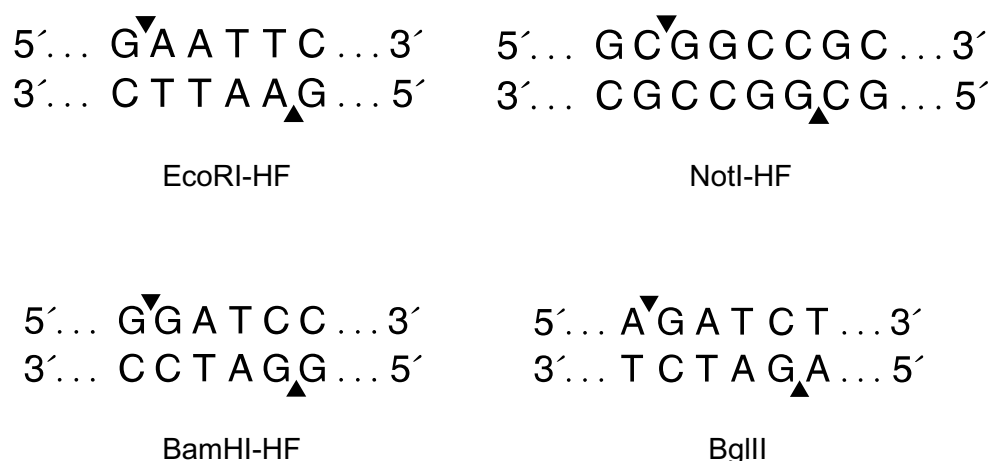*Figure 11. Recognition and cut sites of restriction enzymes: EcoRI-HF, NotI-HF, BamHI-HF and BglII (New England Biolabs Inc., n.d.).*

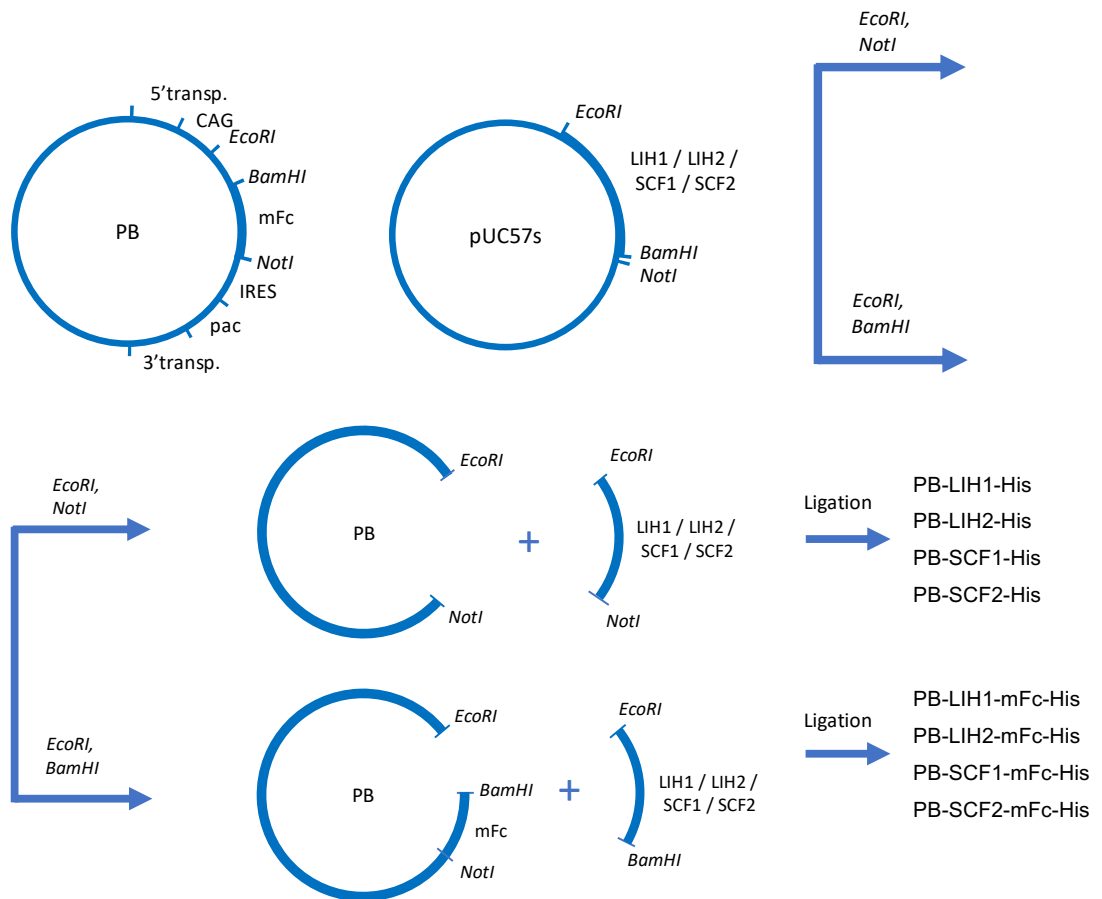**Figure 12.** *Schematic overview of subcloning procedures of Fab, scFv-mFc and IgG (with mFc) antibody constructs with RE sites.*
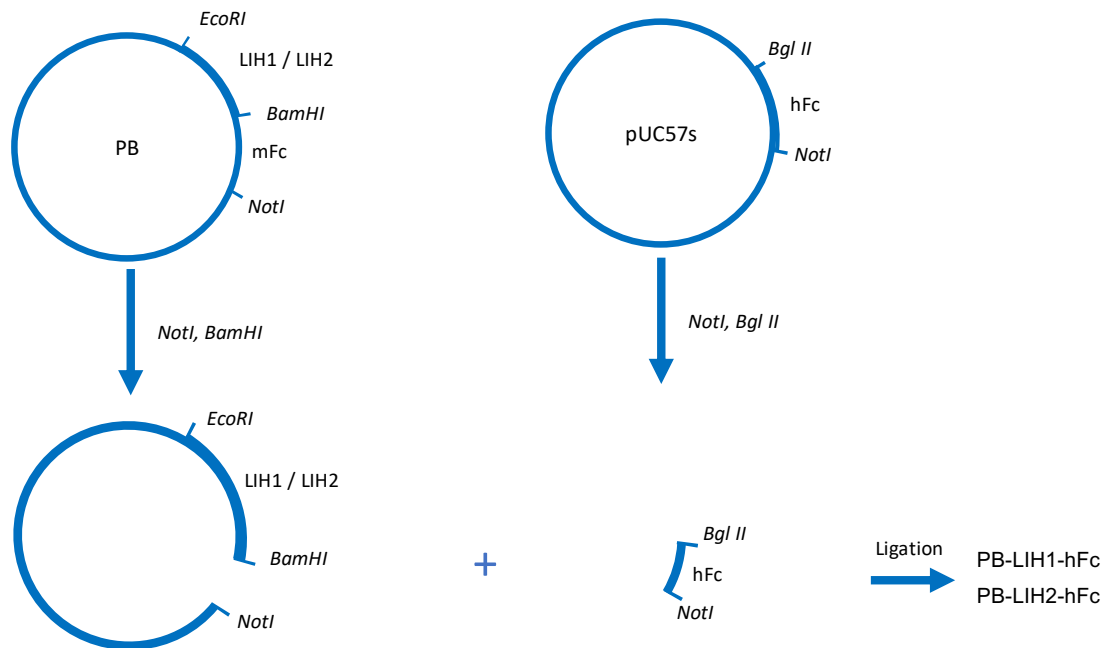

Figure 13. *Schematic overview of subcloning procedures of IgG (with hFc) antibody constructs with RE sites.*

1µg of each ligate was transformed into *E. coli* DH5α cells by the heat shock method, which were then grown on A-100 LB agar plates o/n at +37°C according to the previously described protocol. Following the same protocol, two individual colonies of each plasmid were collected and grown in the 5 ml LB solution with 10 µl of ampicillin (+37°C, o/n on shaker). The final plasmid DNA was extracted on the following day by PureYield™ Plasmid Miniprep System (Promega) kit and its protocol. In particular, PureYield™ protocol includes the endotoxin wash step, which removes endotoxin contaminants from the purified plasmid DNA for further eukaryotic transfection.

After the purification of the final DNA plasmids aimed for transfection, test RE-digestions were performed and ran by gel electrophoresis to ensure the correctness of ligation and resulting DNA constructs based on the insert sizes.

4.3 Transfection and cell culture

The cloning and subcloning of antibody constructs resulted in expression vectors, which contain all the necessary components for stable transfection and further cell line selection. Besides the antibody construct sequence fused with polyhistidine tag (absent in hFc constructs), it contains CAG promoter with enhancer elements and puromycin-N-acetyl-transferase gene (*pac*) – a puromycin selection marker. pPB-CAG expression vectors also include the transposon-specific ITR sequences located on both ends of the transposon vector (Figure 12), which are recognized by the PB transposase for subsequent gene transfer into a host cell genome during stable transfection.

The day before the transfection, HEK293F cells were plated onto the 6-well plates in complete Dulbecco's Modified Eagle Medium (cDMEM; Gibco™), which is supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Corning™), overnight in +37°C in 5% CO_2. 10 antibody constructs described in section 4.1 were transfected the next day into HEK293F cells by lipofection method using FuGENE®6 Transfection Reagent (Promega). In this method, the cationic lipids bind to negatively charged plasmid DNA facilitating the nucleic acid delivery into the mammalian cell (Jacobsen, 2004).

6 µl of transfection reagent was diluted in 120 µl of serum-free medium (DMEM) and mixed. 1,2 µg of DNA and 0,12 µg of PB transposase DNA was then added to the reaction mix for formation of the complex for total 20 min (protocol in Appendix 10). The mixture was then added directly to the cells for further incubation for 3-4 days.

As previously mentioned, pPB-CAG expression vectors contain puromycin selection marker. Cell line selection was carried out by incubating cells with gradually increasing puromycin concentrations in series (typically 0, 2, 4, 6, 8, 10, 15, 20, 30, 40, 60, 80, 100,

120 µg/ml) and selecting surviving cells cultured in 6-well plates. The interval of all further passages of adherent monolayer cells was 3-4 days, reaching approx. 90% confluence in T25 (25 cm^2) and T75 (75 cm^2) cell culture flasks (Greiner Bio-One$^®$).

After the puromycin selection process, stably transfected HEK293F cells were transferred to grow in suspension (+37°C, 5% CO_2, 120 rpm) in protein-free OptiCHO™ Medium (Gibco™) optimal for high performance in recombinant protein production. At this stage, the test monolayer protein production (T25, 5 ml) was also carried out and collected for further analysis.

The suspension cells were passaged with an interval of 3-4 days (passages to 0,4 x 10^6 cells/ml) with the purpose of growing the cell mass within the following volumes: 30, 70, 150 and eventually 300 ml. Depending on the results of the test production, the final protein production was carried out in the volumes of 150 ml or 300 ml in OptiCHO™ medium for 5 days (1,0 x 10^6 cells/ml; +33°C, 5% CO_2, 120 rpm). More detailed data on the production volumes and rates are found in Table 4 of section 5.4.

4.4 Protein purification and affinity chromatography

The recombinant antibodies and antibody fragments produced in this work are secreted proteins, implying that the supernatant of centrifugated cell suspension contains the proteins of interest. One of the first stages in the purification process of the production cell suspensions was the collection of supernatant after centrifugation (3700 rpm) and its filtration with the 0.22 µm vacuum filter (Steritop$^®$, Millipore), which removes possible contamination, such as microorganisms and other unwanted particles. The following protein purification steps include affinity chromatography and dialysis membrane tubing. Depending on the construct, different volumes of protein solutions were purified, varying between 50 and 300 ml. More detailed data on the volumes of purified proteins and the yields is found in Table 4 of section 5.4.

The constructs containing polyhistidine-tags (His-tags) on the C-terminus were purified by immobilized metal affinity chromatography (IMAC). His-tagged recombinant proteins were loaded through the affinity column (Protino$^®$ Ni-NTA 1ml, Macherey-Nagel) and captured by interactions with nickel ions (Ni^{2+}). The purification was carried out by pump with the protein solution circulating through the column overnight. The wash step and protein elution were performed on the following day by running imidazole buffers with increasing concentrations (5-500 mM) through the affinity column (Appendix 11). Imidazole competes with the interaction between His-tag and immobilized Ni^{2+} ions, thereby

releasing the target protein (Figure 14). The His-tag was not removed afterwards as it is expected not to affect the binding properties of the antibody constructs.
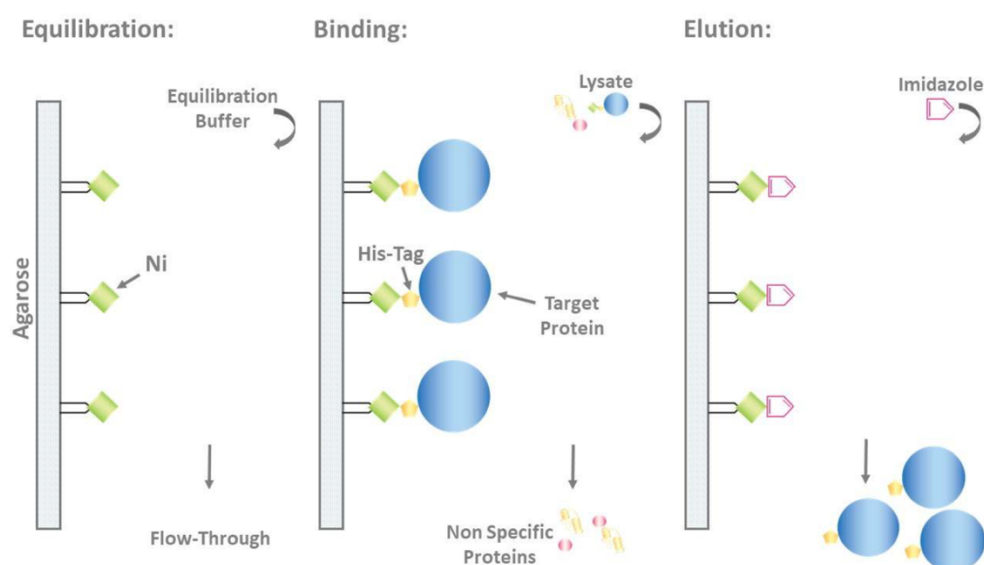

Figure 14. Principle of immobilized metal affinity chromatography (IMAC). His-tag of the target protein binds nickel ion in the column, where the protein is then eluted by imidazole, which competes with the protein for binding. Figure retrieved from GoldBio, 2018.

The antibody variants with human hFc region do not contain His-tag and instead were purified by other methods. LIH1-hFc construct was purified by means of another protein, consisting of RBD region of SARS-CoV-2, mFc region and His-tag (RBD-mFc-His6, produced in-house). The principle of this purification utilized the binding properties and interaction between RBD and the recombinant antibody. The His-tagged RBD-mFc-His6 was first loaded through the previously described affinity column (Ni^{2+}). The following loading of LIH1-hFc resulted in column-bound protein of interest. The elution of LIH1-hFc was carried out with 7M Urea and 1M NaCl (Appendix 12).

The purification of LIH2-hFc was performed by protein A affinity chromatography, which utilizes specificity and strong binding affinity of protein A and Fc region of IgG. The protein solution was loaded through the protein A Sepharose affinity column (HiTrap Protein A HP 1ml, Cytiva) by pump overnight and eluted with 0.1M citric acid and 1M Tris-HCl (Appendix 13).

Upon analyzing the results of SDS-PAGE, which is described in the following section, the IMAC fractions were selected and purified by dialysis membrane tubing. Dialysis tubing is a semi-permeable cellulose membrane used to remove low-molecular-weight compounds from the target protein solution, such as imidazole molecules used in protein

elution. The IMAC fractions were placed into the dialysis tubing (MWCO 6-8 kDa, Spectrum™ Labs), sealed and placed into PBS buffer (100 ml per 1 ml of protein fractions) overnight on the magnetic stirrer. The concentrations of purified proteins were measured with Nanodrop 2000 spectrophotometer (Thermo Scientific™) and aliquoted for storage in -80°C.

4.5 SDS-PAGE and Western blot analysis

The analysis of test productions, as well as final purifications of the recombinant antibodies and antibody fragments, was performed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. In particular, SDS-PAGE allowed to assess the purity, size and relative yield of produced proteins. In addition, Western blotting demonstrated the results of test productions as well as the correctness of the expressed protein constructs.

SDS-PAGE is a commonly used analytical technique aimed to separate proteins based on their molecular weight. SDS is a detergent, which denaturates proteins by binding to the protein backbone, resulting in unfolded linear polypeptide chains with an evenly distributed negative charge. Upon application of an electric field, the proteins migrate towards the anode at different distances, depending on their molecular weight. Depending on the goals of analysis, a reducing agent (dithiothreitol - DTT) was also added to certain samples in order to investigate the proteins' expected properties and structure. DTT is a commonly used reagent used for reducing disulfide bonds between and within biological molecules when oxidized.

In SDS-PAGE, 10 µl of Laemmli sample buffer (LSB), which contains SDS, or LSB+DTT mix was added to the samples (40 µl) and boiled in Eppendorf Tubes® for 3 min to promote the protein denaturation. The samples (10 µl each) were loaded into Any kD Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) and run in a buffer at 180V for approx. 35 min. The gels were fixed and stained by Coomassie dye (PageBlue™ Protein Staining Solution, Thermo Scientific™) overnight or proceeded with Western blot protocol.

In Western blot, the proteins in gel were blotted onto the nitrocellulose membranes by electrophoretic transfer in transfer buffer (150V, 25 min). To prevent non-specific binding of the antibodies, the membrane was blocked by 5% milk on a shaker. The membrane was then incubated at +4°C overnight with primary antibodies diluted in milk: 6-His-Tag monoclonal antibodies (1:5000, Invitrogen) for His-tagged constructs and anti-human Fc antibodies (1:10000; Sigma) for hFc constructs. On the following day, the membrane

was rinsed with wash buffer (TBST) and incubated with secondary anti-mouse HRP polyclonal antibodies (Jackson ImmunoResearch; 1:10000) for 4 hours on a shaker at a room temperature. Horse Radish Peroxidase (HRP)-conjugated secondary antibodies bind specifically to the primary mouse IgG Fc region, allowing immunodetection of proteins on the membrane. The reaction of HRP with added chemiluminescent substrates (Western Lightning™ Pro, PerkinElmer) results in luminescent signal detected and visualized by a luminometer.

4.6 Pseudovirus neutralization assay

4.6.1 Assay principle

Pseudoviruses represent useful research tools used to investigate highly infectious and pathogenic viruses, such as SARS-CoV-2, as they can be handled safely in BSL-2 conditions, unlike natural viruses. They are synthetically modified viruses consisting of a parental viral core, typically derived from rhabdovirus or retrovirus, and a surface envelope glycoprotein derived from a virus of interest (Neerukonda, 2021).

The parental viral genome is modified to lack the essential genes required for replication in a host cell, which results in only single viral infection cycle without the ability to replicate further. The genome of pseudovirus also contains a reporter gene coding for luciferase (or fluorescent protein), which is a key quantifying element in this bioassay (Neerukonda, 2021).

In the pseudovirus neutralization assay for SARS-CoV-2 within this study, a pseudovirus expressing SARS-CoV-2 S glycoproteins binds ACE2 receptors of the host cells, resulting in S protein conformational change necessary for virus fusion with the cell (Wang, 2022). Upon successful host cell entry, pseudovirus genome is released and reporter luciferase gene is expressed. The luciferase expressed by the cells machinery can be detected upon reaction with the reagents resulting in luminescence signals, which are measured by a luminometer and correlated with the level of viral entry (Figure 15). Thus, the viral entry mediated by S protein of SARS-CoV-2 can be measured via luciferase activity (Wang, 2022).

In this assay, the cells are incubated with pseudovirus and various dilutions of neutralizing antibodies of interest. The antibody constructs produced in this study specifically bind RBD of pseudovirus S protein, preventing its entry to the host cell. The neutralization results in a decrease of the luminescence signal, as a subsequent genome release and reporter gene expression are blocked (Figure 16).
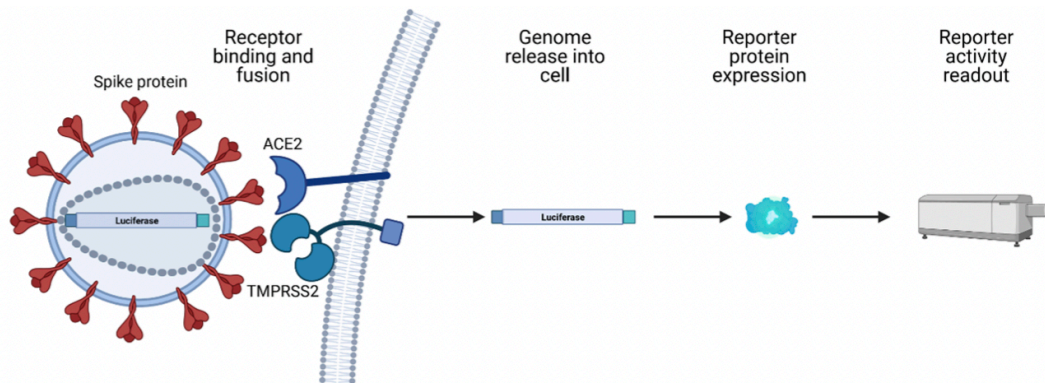
Figure 15. *The principle of SARS-CoV-2 pseudovirus neutralization assay. Upon binding the ACE2 receptor, pseudovirus genome is released into the cell, where the expressed luciferase allows the quantifying of the luminescence signal. Figure adopted from Steward, 2021.*
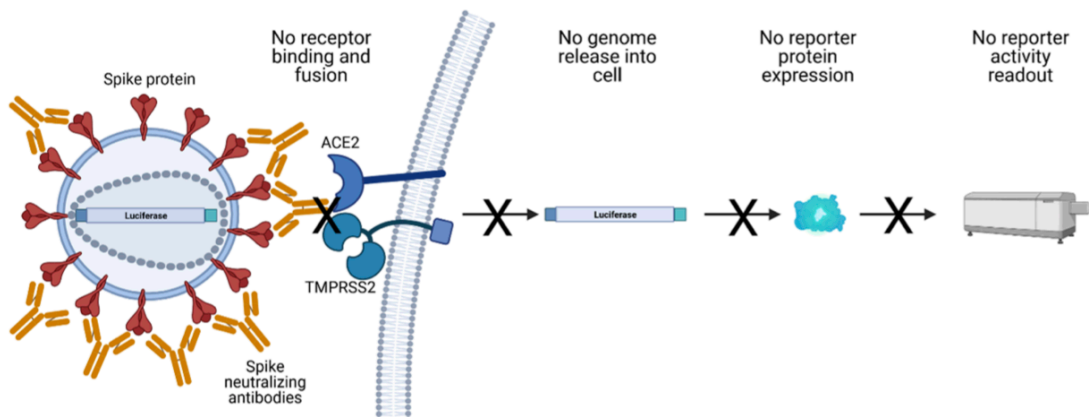

Figure 16. *The principle of SARS-CoV-2 pseudovirus neutralization assay. Neutralizing antibodies prevent pseudovirus from entering the cell via ACE2 receptor, which results in the absence/reduction of luminescence signal. Figure adopted from Steward, 2021.*

4.6.2 Workflow

Lentivirus based pseudoviruses, which introduce S protein of Wuhan, Beta or Delta SARS-CoV-2 variants on their envelope were used in this work. The pseudoviruses and ACE2 overexpressing HEK293T cells were generated previously in HEK293T cells transfected with plasmids that produce lentiviral structural proteins, spike protein and ACE2.

The ACE2 overexpressing HEK293T cells were plated onto 96-well plate (2×10^4 cells/well) in 100 µl of cDMEM and incubated with 37,5 µl of pseudovirus (Wuhan, Delta or Beta variants) and serially diluted antibodies (from 1 µl/ml to 0.001 ng/ml) at +37°C (5% CO_2) for 2 days. After the incubation, the liquid was removed from the plate and replaced with a 50 µl mix of PBS, Renilla-Glo® Luciferase Assay buffer and substrate

(Promega). The buffer promotes cell lysis and the coelenterazine substrate provides the bioluminescent reaction catalyzed by *Renilla* luciferase expressed in the incubated cells (Figure 17).
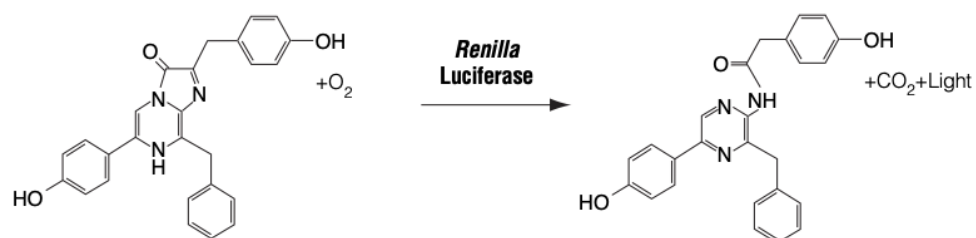
Figure 17. *Bioluminescent reaction catalyzed by Renilla luciferase. Figure retrieved from Promega, 2011.*

The measurement of the luminescent signal was performed after a minimum 12 min of reaction time to achieve an optimal signal according to the manufacturer protocol. The generated luminescence light absorbance was measured by a detector (Hidex) as luminescence counts.

The results obtained from pseudovirus neutralization assay as luminescence counts were converted into the percentage of luminescence at a given logarithmic concentration of antibody. The data was plotted by a dose-response curve in GraphPad Prism v9.

5 RESULTS AND DISCUSSION

Recombinant antibodies and antibody fragments are essential and promising biomole-cules for use in biotechnology and therapeutics. They represent highly specific tools for targeting certain biological targets, such as cancer-associated molecules or pathogen antigens (Santos, 2018).

This thesis work aimed to produce 10 recombinant anti-SARS-CoV-2 monoclonal anti-bodies and antibody fragments by carrying out the DNA cloning and subcloning, expres-sion in mammalian cell line and purification. The goal was to perform bioassays to assess the functionality of the produced Abs and evaluate the overall success of the used ex-pression approaches. This section presents and discusses the results along with the characterization of the mAb constructs produced in this work.

5.1 DNA cloning

The 10 antibody constructs were based on gene sequences of two SARS-CoV-2 anti-bodies, REGN10933 (imdevimab) and REGN10987 (casirivimab) (Regeneron Pharma-ceuticals) (Baum, 2020a). The original gene sequences of SARS-CoV-2 antibodies were obtained from PBD (ID: 6XDG) and the antibody constructs were designed by Olli Ritvos.

The LIH1 and SCF1, LIH2 and SCF2 inserts based on Regeneron REGN10987 and REGN10933 sequences, respectively, were successfully subcloned from commercially synthesized pUC57-simple cloning vectors into the piggyBac (PB) expression vectors. Restriction enzyme (RE) analysis was carried out for all of the constructs to see if correct sizes for insert and vector fragments were found. Figure 18 shows a representative result for two of the constructs, showing two identical clones cut by two REs. Both were found to have inserts of expected sizes.
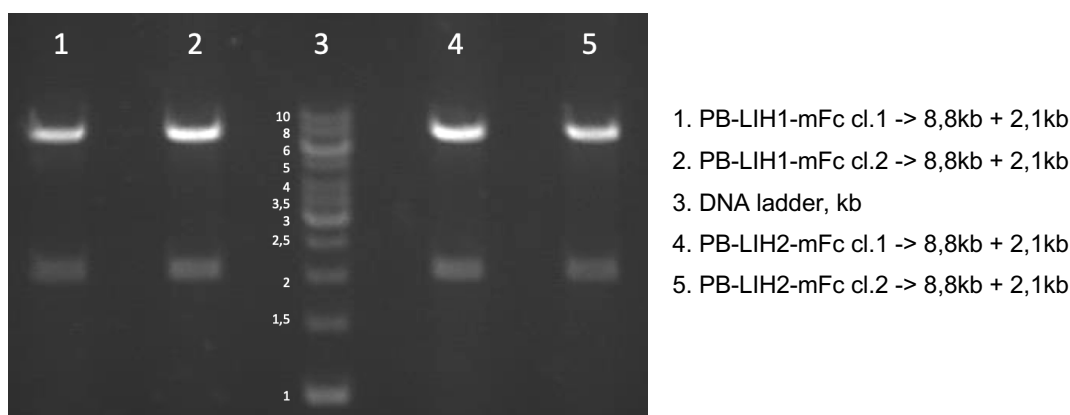
Figure 18. *Test restriction digestion of PB-LIH1-mFc and PB-LIH2-mFc clones by EcoRI-HF + BamHI-HF RE resulting in PB-mFc (~8,0 kb (PB) + ~0,8kb (mFc) = ~8,8 kb) and LIH1 or LIH2 (~2,1 kb) bands. 1,0 % agarose gel run with Midori Green, 100V.*

5.2 Protein expression

The PiggyBac (PB) transposon system was used for transfection and expression of the antibody constructs in HEK293F cell line (a variant of HEK293 cells adapted to commercial medium and suited for suspension culture). This system includes PB expression vectors, which are widely used in biotechnology providing highly efficient transposition of the gene of interest located between ITRs into the rather random location in cell genome. This results in a stable cell line and stable gene expression allowing the long-term production of recombinant protein. The use of CAG promoter, in turn, contributed to sufficient protein yields, giving high transcription efficiency by preventing methylation of the promoter (with the help of CpG island) and keeping it active (Dou, 2021).

The choice of the host cells relied on cell line characteristics and previous experience showing more efficient recombinant protein production in HEK293F cells compared to CHO cells. HEK293 cells are generally more suitable for research purposes, which require relatively small amounts of the product produced in a short time. They provide high quality and cost-efficient mammalian protein expression within a shorter time, compared to CHO cells. The conditions and reagents previously used by the research group were optimized for the HEK293F cells. In particular, higher culture temperature during production phase and CHO culture medium were customized for expression in HEK293F cells.

Antibody fragments, such as Fabs and scFvs can be also efficiently expressed in microorganisms (most frequently *E. coli*), offering significant advantages for the production process such as low cost, high yields and shorter time of production (Hu, 2017; Kang, 2020). However, *E. coli* cannot provide essential PTMs, which are important for Ab stability, especially for the constructs possessing Fc region. In particular, glycosylation in

the Fc region affects the stability of Abs. In contrast, mammalian cells provide more natural PTMs and protein folding conditions for Abs and Ab fragments (Zheng, 2011).

The presence of the puromycin selection marker gene (*pac*) allowed to establish a stable cell line for long-term production as well as for future use of the cells that were frozen. The target concentration of puromycin for the cell line selection was 100-120 µg/ml, which was successfully reached in all constructs. This ensured sufficiently high levels of expression of the proteins encoded within the transposon.

In addition, prior to transferring the monolayer cells to suspension culture, test productions on monolayer cells were carried out in small volumes (5 ml, protein-free medium). The samples taken from precipitated cells and supernatant (3 days culture) were analyzed with Coomassie stained SDS-PAGE and detection by Western blotting. This step ensured secretion of the proteins from the cells into the culture medium as well as the correctness of protein conformation and sizes before proceeding with a larger production. Monolayer test production also showed approximate expression levels, which may be lower than in suspension culture due to the cell density and better conditions.

Test productions have shown positive results with variable degrees of secretion from the cells. However, the exception was SCF2-8His construct, which showed abnormal results in Western blotting (Figure 19). The bands indicate possible dimerization of scFvs, which may be caused due to cysteine amino acids within the VH and VL variable domains. The cysteines apparently create disulfide bridges between scFvs, which seem to be reduced by DTT according to Figure 19. This issue, however, seems to be overcome in the case of SCF2-mFc-6His construct, which contains the same variable domains. The reason

1. HEK293F SCF1-his cells monolayer +DTT
2. HEK293F SCF1-his cells monolayer −DTT
3. HEK293F SCF1-his sup monolayer +DTT
4. HEK293F SCF1-his sup monolayer −DTT
5. HEK293F SCF1-his sup suspension +DTT
6. HEK293F SCF1-his sup suspension −DTT
7. Mw
8. HEK293F SCF2-his cells monolayer +DTT
9. HEK293F SCF2-his cells monolayer −DTT
10. HEK293F SCF2-his sup monolayer +DTT
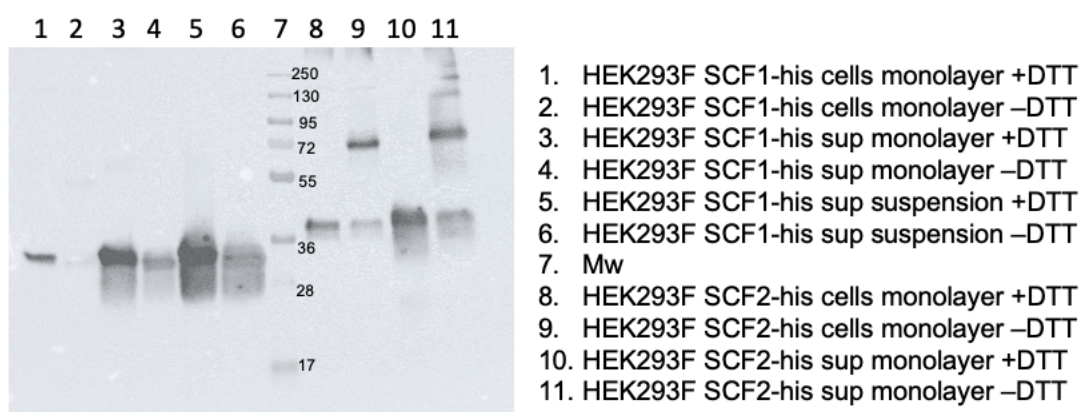11. HEK293F SCF2-his sup monolayer −DTT

Figure 19. *Test production of SCF1-8His (~33 kDa) and SCF2-8His (expected size ~33 kDa). SDS-PAGE, Western blotting: anti-his 1:5000, anti-mouse-HRP 1:10 000. SCF2-8His bands show possible undesired dimerization, which is reduced in +DTT samples.*

apparently lies in the presence of Fc region, which displaces and stabilizes scFvs avoiding the formation of undesired bonds.

5.3 Protein purification

Purification of the proteins possessing His-tags was performed by IMAC, a commonly used and cost-efficient purification method utilizing a strong affinity interaction between Ni^{2+} ions and polyhistidine tag. IMAC purification results of each antibody construct were analyzed by running SDS-PAGE with the samples taken from the corresponding purification steps: *in* (IN), *flow-through* (FT), *wash* (W) and *elution* (E) fractions. The results demonstrated the correctness of protein conformation under reducing conditions (DTT) and sufficient purity of the product. The relative concentrations were also estimated by running bovine serum albumin (BSA) of several concentrations alongside the samples.

Figure 20 represents an example of performed IMAC purification, showing high expression levels and purity as well as correct band sizes resulted from the reduction of disulfide bonds between HC-mFc and LC. SDS-PAGE-displayed molecular weights frequently look larger than the expected size due to protein PTMs, such as glycosylation.

Figure 20. IMAC fractions from purification of LIH2-mFc-6His, MW (kDa), and BSA samples. Coomassie blue stained SDS-PAGE gel, all samples undiluted and reduced. Bands demonstrate reduction of disulfide bonds between HC-mFc (~57 kDa) and LC (~26 kDa).

The antibody variants possessing human Fc region (hFc) were purified by other techniques as they do not contain His-tag. In particular, LIH2-hFc was successfully purified by protein A affinity chromatography (Figure 21), which utilizes strong binding affinity interaction between protein A and Fc region.
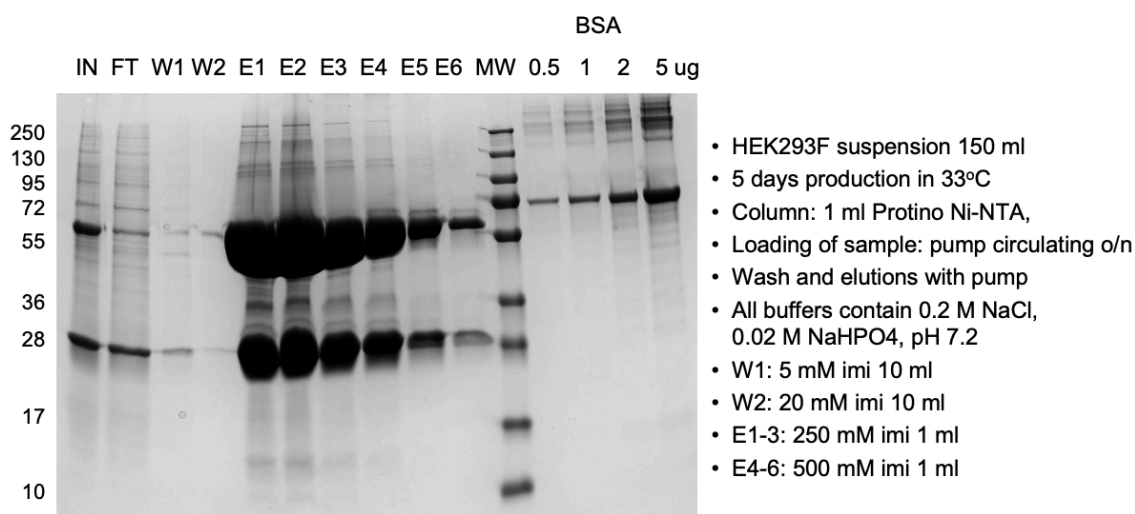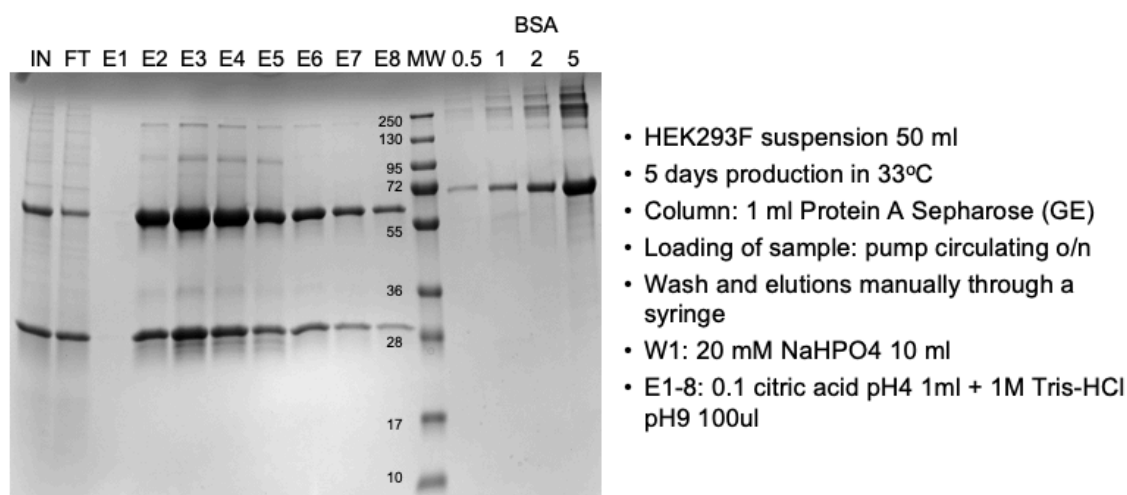
Figure 21. Fractions from protein A purification of LIH2-hFc, MW (kDa), and BSA samples. Coomassie blue stained SDS-PAGE gel, all samples undiluted and re-duced. Bands demonstrate reduction of disulfide bonds between HC-hFc (~55 kDa) and LC (~26 kDa).

LIH1-hFc construct was purified by a distinct experimental method. This purification used RBD-mFc-His6 recombinant protein (in-house produced), which consists of RBD region of SARS-CoV-2, mFc region and His-tag. In this approach, His-tag binds Ni^{2+} in IMAC column, while LIH1-hFc is captured by its affinity to RBD (Figure 22). This method demonstrated its eligibility and sufficient purification results, although with relatively low yield. The approach additionally affirmed the specificity and affinity properties of LIH1-hFc against RBD of SARS-CoV-2.
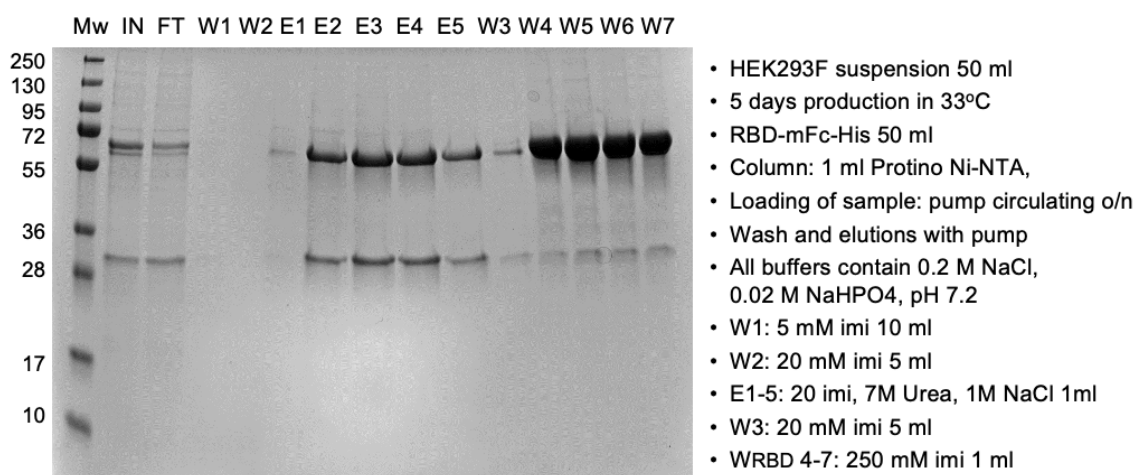

Figure 22. Purification fractions of LIH1-hFc captured in IMAC via RBD-mFc-6His. Coomassie blue stained SDS-PAGE gel, all samples undiluted and re-duced. E1-E5: elution of LIH1-hFc by urea solution; W4-W7: elution of RBD-mFc-6His. Bands demonstrate reduction of disulfide bonds between HC-hFc (~55 kDa) and LC (~26 kDa).

An additional purification step for all proteins was dialysis through a semi-permeable membrane, which removes the high imidazole concentration used in protein elution.

These steps together were enough to achieve sufficiently pure recombinant protein. In addition, similar preparations from our laboratory were run in ion exchange chromatography in a collaboration with Minna Hankaniemi (Tampere University). The results demonstrated satisfactory purity of the samples (data not shown).

Another potential purification method could be size exclusion chromatography, which, however, presents several disadvantages, such as poorer selectivity and capacity along with the loss of protein during multi-stage procedures (Ventouri, 2020).

5.4 Characterization of antibody constructs

In 2020, Hansen et al. have described the generation of a large library of highly effective human neutralizing antibodies, which target RBD of the S protein of SARS-CoV-2. They used humanized mice immunized with a DNA plasmid expressing S protein, and B cells from humans recovering from COVID-19 to obtain a large collection of human antibodies with diverse sequences and binding properties. The selection of the most effective antibodies resulted in a collection of pairs of highly potent antibodies that bind the RBD simultaneously in a non-overlapping manner, thus, do not compete for the binding (Hansen, 2020).

Baum et al. (2020b) focused on four of these non-overlapping antibodies, which were effective against known spike variants at that time. They sequenced and identified light and heavy chains of these antibodies and generated corresponding Fabs to investigate their mechanism of neutralization (Baum, 2020b; Hansen, 2020). They succeeded to select a pair of antibodies that eventually became an effective immunotherapy treatment when used together (Weinreich, 2021b). In particular, REGN10987+REGN10933 antibody cocktail retained the ability to neutralize the viral mutants as its two components can simultaneously bind two distinct and relatively conserved epitopes within RBD. This property could also protect against the virus escape mutants that cause antibody resistance, which, in turn, can emerge in response to single-antibody treatments and subsequent selective pressure (Baum, 2020b; Hansen, 2020).

The antibody constructs produced in this work are based on REGN10987 and REGN10933 sequences, which were redesigned and codon-optimized to express IgG, Fab, scFv and scFv-Fc antibody fragments. Antibodies of different conformations that can bind two distinct non-overlapping epitopes within RBD of the SARS-CoV-2 spike

protein present a promising biotechnology tool, which can be potentially used in diagnostics and research, considering the fact that RBD is a key viral site responsible for binding ACE2 receptors.

Table 4 provides an overview of the produced constructs with general information on their sizes and resulted expression levels. Coomassie blue staining and Western blotting were also carried out on SDS-PAGE gels (Figures 23-26) to provide a visual summary of all purified constructs under denaturing conditions (high temperature) as well as reducing conditions (DTT). A more detailed description of the constructs is provided in Section 4.1.

Table 4. General information on produced and purified antibody constructs.

Construct (type)	Size, ~aa	Size, ~kDa	Production/ purification volume	Yield, Yield/litre of cell culture
LIH1-8his (Fab)	498 aa	55 kDa (HC = 29 kDa; LC = 26 kDa)	300 ml/ 150 ml	**6 mg, 40 mg/l**
LIH2-8his (Fab)	494 aa	55 kDa (HC = 29 kDa; LC = 26 kDa)	300 ml/ 150 ml	**6,4 mg 42,6 mg/l**
LIH1-mFc-6his (IgG)	744 aa	83 kDa (mFc = 27 kDa) Full IgG = 166 kDa	300 ml/ 150 ml	**18,5 mg, 123,3 mg/l**
LIH2-mFc-6his (IgG)	740 aa	82 kDa Full IgG = 164 kDa	300 ml/ 150 ml	**24,4 mg, 162,7 mg/l**
LIH1-hFc (IgG)	728 aa	81 kDa (hFc = 26 kDa) Full IgG = 162 kDa	150 ml/ 50 ml	**1 mg, 20 mg/l**
LIH2-hFc (IgG)	724 aa	81 kDa Full IgG = 162 kDa	150 ml/ 50 ml	**3,5 mg, 70 mg/l**
SCF1-mFc-6his (scFv-Fc)	549 aa	61 kDa Full IgG = 122 kDa	300 ml/ 150 ml	**15 mg, 100 mg/l**
SCF2-mFc-6his (scFv-Fc)	544 aa	61 kDa Full IgG = 122 kDa	300 ml/ 100 ml	**4,4 mg, 44 mg/l**
SCF1-8his (scFv)	303 aa	34 kDa	150 ml/ 100 ml	**14,8 mg, 148 mg/l**
SCF2-8his (scFv)	298 aa	33 kDa	No production	-

The antibody fragments generally have lower stability than full-length antibodies. In particular, Fabs of IgG typically become less stable and more sensitive to denaturation when Fc region is removed (Ionescu, 2008). Other forms of antibody fragments, which are naturally smaller, are also commonly considered less stable. In particular, the glycosylation of Fc region in complete Abs plays an important role in correct protein folding and stability, while unglycosylated Ab fragments typically demonstrate lower stability and vulnerability to aggregation (Ma, 2020). The Fc domain fused with Ab fragments also provides the dimerization features, which enhances their binding properties.

1. Mw (kDa)
2. 293F PB-LIH1-8His +DTT
3. 293F PB-LIH1-8His –DTT
4. 293F PB-LIH2-8His +DTT
5. 293F PB-LIH2-8His –DTT
6. 293F PB-LIH1-mFc-6His +DTT
7. 293F PB-LIH1-mFc-6His –DTT
8. 293F PB-LIH2-mFc-6His +DTT
9. 293F PB-LIH2-mFc-6His –DTT
10. 293F PB-LIH1-hFc +DTT
11. 293F PB-LIH1-hFc –DTT
12. 293F PB-LIH2-hFc +DTT
13. 293F PB-LIH2-hFc –DTT
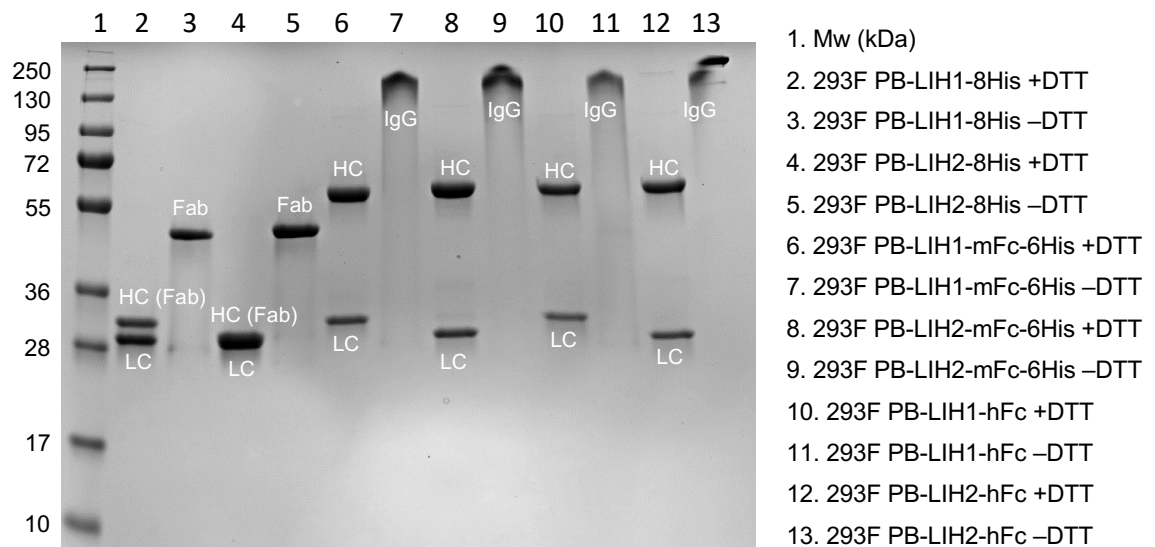
Fig ... *ed SDS-PAGE gel. All diluted to*
0, ... *C and HC; Fc and*
F ... *le 4.*

(kDa)
PB-LIH1-8His +DTT
PB-LIH1-8His –DTT
PB-LIH2-8His +DTT
PB-LIH2-8His –DTT
PB-LIH1-mFc-6His +DTT
PB-LIH1-mFc-6His –DTT
PB-LIH2-mFc-6His +DTT
PB-LIH2-mFc-6His –DTT

10. Mw (kDa)
11. 293F PB-LIH1-hFc +DTT
12. 293F PB-LIH1-hFc –DTT
13. 293F PB-LIH2-hFc +DTT
14. 293F PB-LIH2-hFc –DTT

*Figure 24. LIH constructs. Western blotting detection: **2-9**: anti-His 1:5000, anti-mouse-HRP 1:10 000; **11-14**: anti-Fc 1:10 000, anti-mouse-HRP 1:10 000. Load 10ul/well, 3 ug/well. As His-tag is located within HC, LC are not visible. Sizes of mAb elements found in Table 4.*
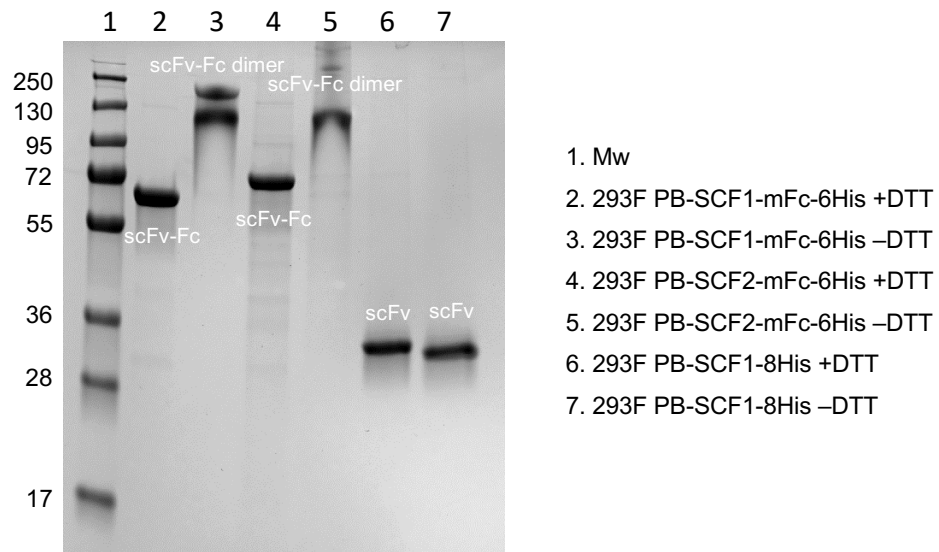
1 2 3 4 5 6 7

250
130
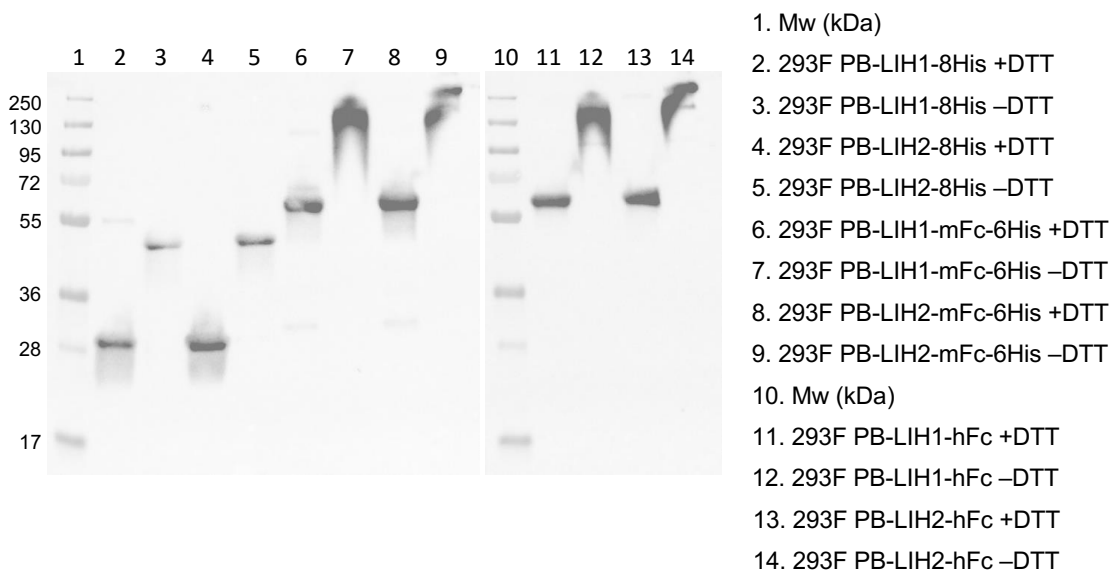95
72
55
36
28
17

scFv-Fc dimer
scFv-Fc dimer
scFv-Fc
scFv-Fc
scFv   scFv

1. Mw

2. 293F PB-SCF1-mFc-6His +DTT

3. 293F PB-SCF1-mFc-6His –DTT

4. 293F PB-SCF2-mFc-6His +DTT

5. 293F PB-SCF2-mFc-6His –DTT

6. 293F PB-SCF1-8His +DTT

7. 293F PB-SCF1-8His –DTT

Figure ... *e blue stained SDS-PAGE gel.* *Load 1* ... *ulfide bonds between Fc and Fc re-sulting* ... *found in Table 4.*

250
130
95
72
55
36
28
17

1. Mw

2. 293F PB-SCF1-mFc-6His +DTT

3. 293F PB-SCF1-mFc-6His –DTT

4. 293F PB-SCF2-mFc-6His +DTT

5. 293F PB-SCF2-mFc-6His –DTT

6. 293F PB-SCF1-8His +DTT

7. 293F PB-SCF1-8His –DTT

Figure 26. SCF constructs. Western blotting detection anti-His 1:5000, anti-mouse-HRP 1:10 000. Load 10ul/well, 3 ug/well. Sizes of the elements found in Table 4.

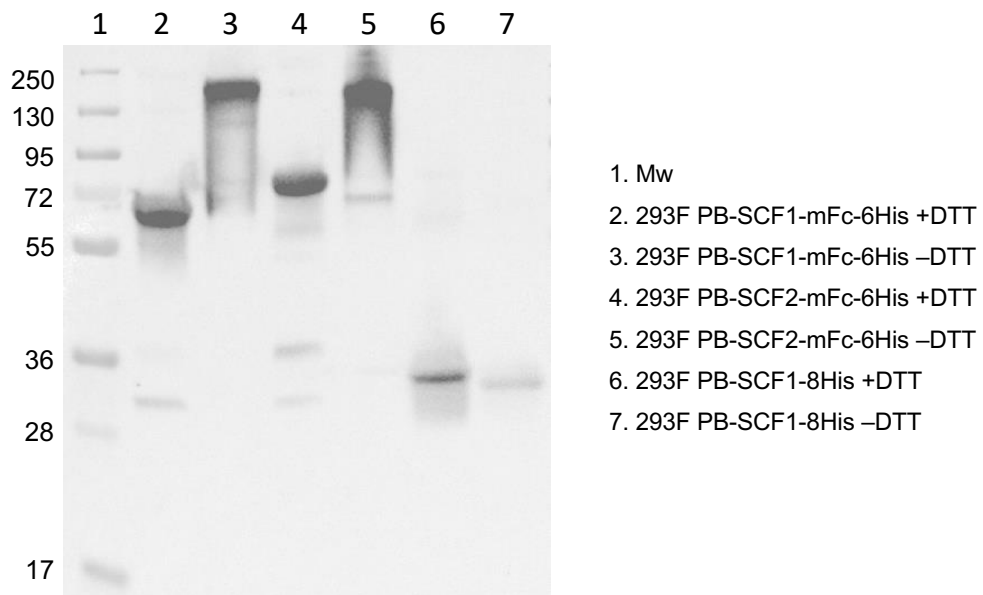LIH constructs produced in this work represent Fab and whole IgG Ab constructs expressed by tricistronic vector. LIH comprise the IRES-mediated tricistronic vector, which can express three genes separately under the control of one promoter. Tricistronic constructs allow the expression of genes in desired proportions, which is important for chain dimerization into functional antibody fragments and the prevention of aggregation. In general, the tricistronic approach for antibody expression is beneficial for satisfactory yields and product stability (Yeo, 2018).

ScFv is a small Ab fragment consisting of the antigen-binding variable domains of heavy and light chains of an Ab, which are connected by a flexible peptide linker. This structure provides high specificity and affinity to the antigen targets while having a smaller molecular size. ScFvs also require relatively few PTMs, which is beneficial for the production process (e.g. the possibility to produce in *E. coli*), although negative for the stability, which is lower than in Fabs. Nevertheless, the poor stability of scFvs can be improved by sequence modifications and protein engineering (e.g. point mutations or addition of stabilizing sequences) (Ma, 2020). In general, scFvs represent promising biomolecules for treatment, diagnostic and research use, having such advantages as low production costs, small size, and potential for genetic fusion with other molecules (Satheeshkumar, 2020).

In general, the expression levels of genes essentially depend on how efficiently they are transcribed. There are multiple factors affecting this process. In particular, the yields of Ab and Ab fragments can be increased by the use of heterologous promoters such as CAG, enhancers and appropriate genetic markers (Khan, 2013). According to the protein expression results in Table 4, expression levels were higher for LIH2-mFc-6His, SCF1-8His, LIH1-mFc-6His and SCF1-mFc-6his, which resulted in yields above 100 mg of purified proteins per litre of HEK293F cell culture. The lowest yield resulted from LIH1-hFc construct, which assumably may be caused by the described non-standard purification via RBD-mFc-His6 protein.

Even though the constructs possess rather similar structure of an antibody, the expression levels may also depend on the structural features, folding and possible secretion issues during the expression. Another factor affecting the expression levels might be the used transfection method, where the transposon inserts the genes of interest into the host cell genome in a rather random manner. The locus of insertion might play a role in the degree of gene transcription. Although the cell line selection ensures positive transfection outcomes and sufficient protein expression, the composition of the cell line cannot guarantee high expression levels and yields.

There are numerous studies that have been aiming to explore and optimize the expression and behaviour of mAb and mAb fragments. The stability enhancement of mAb fragments is one of the central subjects studied with different approaches. Kang & Seong (2020) discuss strategies for increasing the stability of antibody fragments via various approaches such as directed evolution, rational engineering and computational technologies. For instance, the directed evolution via phage display methodology was used by Jespers et al. (2004) to screen for VH region variant resistant to heat denaturation. Rational engineering is another approach used by e.g., Austerberry (2019), which introduced arginine to lysine mutations in scFv fragment resulting in significantly increased aggregation stability (Austerberry, 2019). The recent advances in computational approaches, as well as next-generation sequencing, have also enabled *de novo* engineering of Abs and Ab fragments with optimized sequences and improved recombinant antibody properties (Chevalier, 2017).

5.5 Neutralizing properties

The ability and effectiveness of the produced antibody constructs in neutralizing SARS-CoV-2 were modelled in a pseudovirus neutralization assay. It was performed and analyzed together with a medical student Rauno Naves in our research group. In this bioassay, pseudovirus displaying SARS-CoV-2 S proteins carries a reporter luciferase gene, which allows to detect and quantify the ability of the antibody to neutralize the virus and inhibit its cell entry. To evaluate the efficiency of the constructs, the half maximal inhibitory concentration (IC50) was calculated, which measures the concentration of neutralizing mAbs that block 50% of the virus.

In the case of SARS-CoV-2 wildtype Wuhan variant, the results (Figure 27) demonstrate low IC50 values and quite strong neutralization for all of the mAb constructs, with the exception of Fabs (LIH1-8His and LIH2-8His) and scFv (SCF1-8His). As expected, overall mAb neutralization is the highest for Wuhan variant and decreases for Beta and Delta variants (Figures 27-29), although in varying degrees, and even increases for Delta variant compared to Beta neutralization. The lower efficiency of certain mAb constructs against Beta variant (vs Delta) may be explained by significant mutations in Beta RBD, while Delta RBD mutations enhance the specificity and affinity for ACE2 receptor. Studies have demonstrated that especially the Beta variant actively escapes the neutralization by the most mAbs. (Han, 2022; Li, 2021)
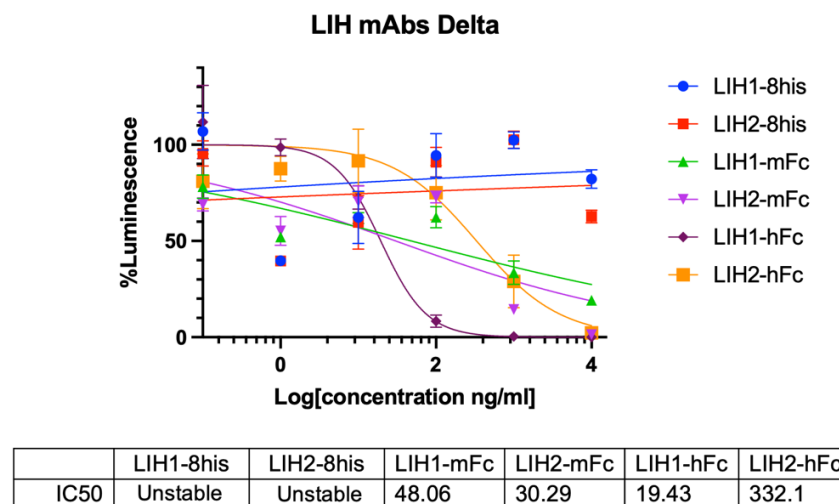
LIH mAbs Wuhan

	LIH1-8his	LIH2-8his	LIH1-mFc	LIH2-mFc	LIH1-hFc	LIH2-hFc
IC50	Unstable	Unstable	4.695	9.629	12.65	18.11

LIH mAbs Beta

	LIH1-8his	LIH2-8his	LIH1-mFc	LIH2-mFc	LIH1-hFc	LIH2-hFc
IC50	Unstable	Unstable	616.2	473.9	12.89	1000

LIH mAbs Delta

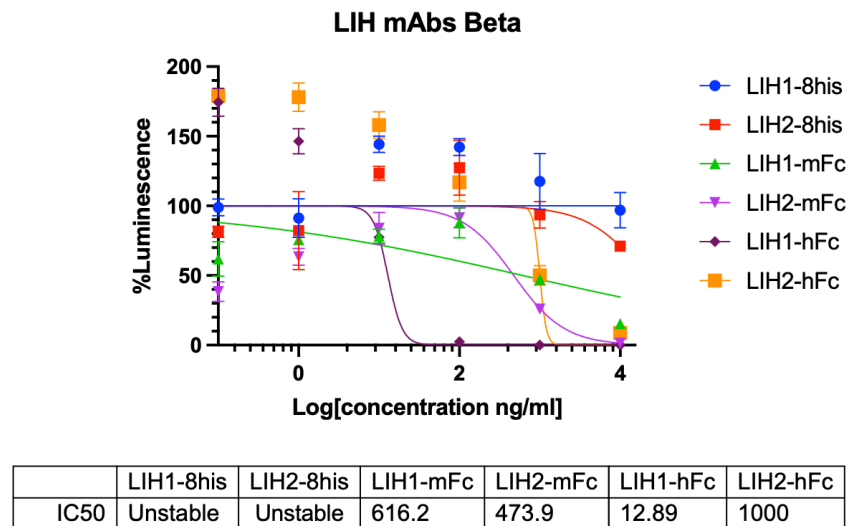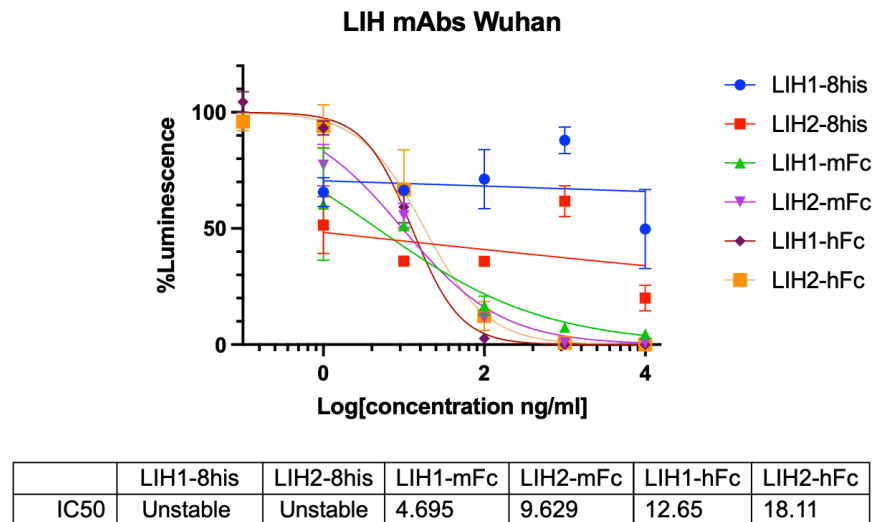|	LIH1-8his	LIH2-8his	LIH1-mFc	LIH2-mFc	LIH1-hFc	LIH2-hFc
IC50	Unstable	Unstable	48.06	30.29	19.43	332.1

***Figure 27.** Pseudovirus neutralization assay results on LIH constructs against S protein of SARS-CoV-2 Wuhan, Beta and Delta variants.*
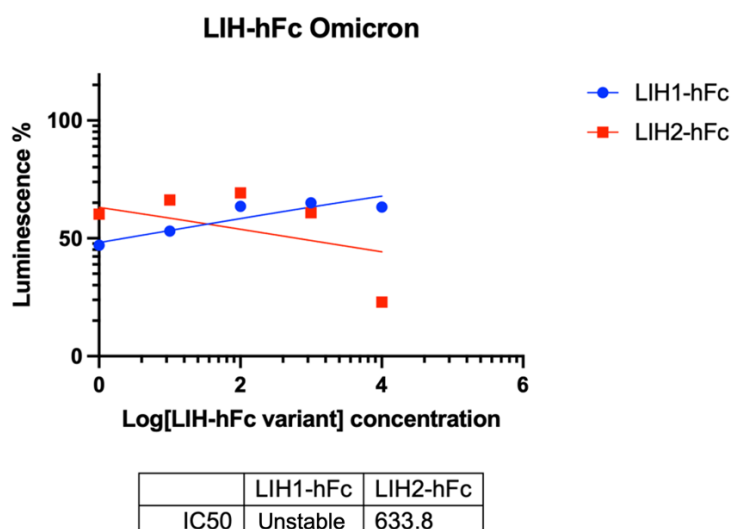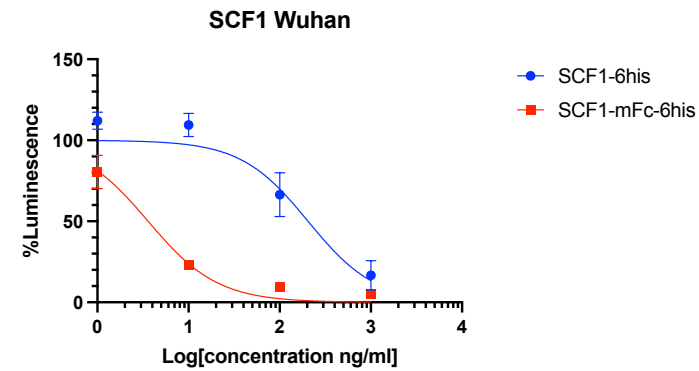
Figure 28. *Pseudovirus neutralization assay results on Fab-hFc constructs against S protein of SARS-CoV-2 Omicron variants.*

Fab-Fc Abs (LIH1-mFc, LIH2-mFc, LIH1-hFc and LIH2-hFc) generally act in a dose-dependent manner as was expected. LIH1-hFc particularly succeeded to retain satisfactory neutralization properties throughout the viral variants. However, the Omicron variant, which was only tested by Fab-hFc antibodies, has fully avoided the neutralization as expected (Figure 28). In turn, Fabs have shown instability in measurements and generally poor neutralization ability, presumably due to the lack of Fc region important for mAb stability and functionality.

SCF1 has demonstrated promising results (Figure 29), where SCF1-mFc mAb has retained the lowest IC50 values throughout the SARS-CoV-2 variants among all the constructs produced and evaluated in this work. SCF1-8His has also demonstrated some mild neutralizing ability, which interestingly increases along with viral variants, which affirms the favorable alignment of antigen-binding variable domains. In the case of SCF1-mFc, the Fc region seems to provide significant beneficial properties for SCF1 scFv fragment, such as stability and dimerization.

SCF2-mFc was also tested in neutralization assay against Wuhan variant, which resulted in absence of neutralization and unstable readings (data not shown). The inability of SCF2-mFc to neutralize the virus was assumed to be caused by SCF2 sequence challenges in variable region alignment, which was a planned risk taken in the design of the constructs. SCF2-mFc was eventually rejected to proceed with further neutralization assays on other SARS-CoV-2 variants.

SCF1 Wuhan

	SCF1-6his	SCF1-mFc-6his
IC50	204.7	3.604

SCF1 Beta
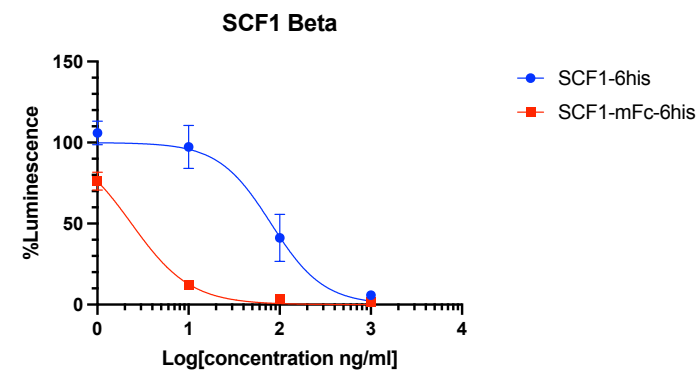
	SCF1-6his	SCF1-mFc-6his
IC50	80.37	2.366

SCF1 Delta
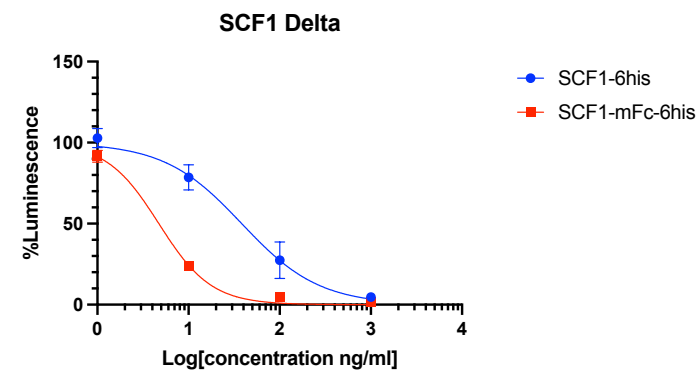
	SCF1-6his	SCF1-mFc-6his
IC50	38.36	4.728

Figure 29. Pseudovirus neutralization assay results on SCF constructs against S protein of SARS-CoV-2 Wuhan, Beta and Delta variants.

The antibody constructs within this thesis work were also tested by other collaborative laboratories. For instance, the colleagues from THL (Finnish Institute for Health and Welfare) have tested a group of antibody constructs that have demonstrated the most promising results in pseudovirus neutralization assay: LIH1-mFc, LIH2-mFc and SCF1-mFc. In particular, they performed a repeated microneutralization assay on multiple SARS-CoV-2 variants (Wuhan, Alpha, Beta, Delta, Mu, Omicron). Preliminary results (data not shown) confirmed our previous results and expectations – SCF1-mFc and LIH2-mFc constructs appear to be the most effective to neutralize most SARS-CoV-2 variants, although they have not demonstrated any efficiency against the Omicron variant.

The anti-SARS-CoV-2 antibodies and antibody fragments produced in this work were also successfully used for immunoassay trials, which affirms their potential usability as a diagnostic and research tool in biotechnology.

6 CONCLUSIONS

This work aimed to produce, characterize and evaluate the virus binding and neutralizing properties of recombinant IgG, scFv, scFv-Fc and Fab monoclonal antibodies and antibody fragments against RBD of SARS-CoV-2. The aim was to compare the neutralization properties of the antibodies variants and assess the overall success of production by the used methods.

The recombinant antibody expression using tricistronic and single chain variable fragments has resulted in satisfactory, though varying protein yields. Some constructs have not gained sufficient stability and anti-SARS-CoV-2 neutralization properties. However, the results are promising with regards to Fc fused mAb fragments. In particular, SCF1-mFc and LIH2-mFc mAbs appear to represent the most successfully produced and effective constructs in the context of SARS-CoV-2 RBD binding and neutralization, although they lose their efficiency against Omicron. The overall trend of the results appears to highlight the contribution of Fc region to mAb stability as well as increased neutralization properties due to Fab-Fc and scFv dimerization ability.

Based on the knowledge obtained from the results of this work, the used mAb production methods will be potentially applied in the future. Although SARS-CoV-2 variants continue to emerge and spread, the obtained biomolecules can be a useful tool for research purposes and also applied for other mAb targets. Various design approaches and optimization of the construct sequences can improve the properties of recombinant antibodies, which represent a significant field of the biopharmaceutical industry and biomedical research.

REFERENCES

Ahmad, Z. A., Yeap, S. K., Ali, A. M., Ho, W. Y., Alitheen, N. B., & Hamid, M. (2012). scFv antibody: principles and clinical application. *Clinical & developmental immunology*, 980250. Retrieved from https://doi.org/10.1155/2012/980250

Alanagreh, L., Alzoughool, F., & Atoum, M. (2020). The Human Coronavirus Disease COVID-19: Its Origin, Characteristics, and Insights into Potential Drugs and Its Mechanisms. *Pathogens, 9*, 331. Retrieved from https://doi.org/10.3390/pathogens9050331

Alfaleh, M. A., Alsaab, H. O., Mahmoud, A. B., Alkayyal, A. A., Jones, M. L., Mahler, S. M., & Hashem, A. M. (2020). Phage Display Derived Monoclonal Antibodies: From Bench to Bedside. *Frontiers in immunology, 11*, 1986. Retrieved from https://doi.org/10.3389/fimmu.2020

Alshrari, A. S., Hudu, S. A., Imran, M., Asdaq, S., Ali, A. M., & Rabbani, S. I. (2022). Innovations and development of Covid-19 vaccines: A patent review. *Journal of infection and public health, 15*(1), 123–131. Retrieved from https://doi.org/10.1016/j.jiph.2021.10.021

Austerberry, J. I., Thistlethwaite, A., Fisher, K., Golovanov, A. P., Pluen, A., Esfandiary, R., van der Walle, C. F., Warwicker, J., Derrick, J. P., & Curtis, R. (2019). Arginine to Lysine Mutations Increase the Aggregation Stability of a Single-Chain Variable Fragment through Unfolded-State Interactions. *Biochemistry, 58*(32), 3413–3421. Retrieved from https://doi.org/10.1021/acs.biochem.9b00367

Azkur, A. K., Akdis, M., Azkur, D., Sokolowska, M., van de Veen, W., Brüggen, M. C., O'Mahony, L., Gao, Y., Nadeau, K., & Akdis, C. A. (2020). Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19. *Allergy, 75*(7), 1564–1581. Retrieved from https://doi.org/10.1111/all.14364

Balasubramanian, S. R. (2016). Comparison of three transposons for the generation of highly productive recombinant CHO cell pools and cell lines. *Biotechnology and bioengineering, 113*(6), 1234–1243. Retrieved from https://doi.org/10.1002/bit.25888

Baldi, L. H. (2007). Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. *Biotechnology letters, 29*(5), 677–684. Retrieved from https://doi.org/10.1007/s10529-006-9297-y

Bates, A. P. (2019). David vs. Goliath: The Structure, Function, and Clinical Prospects of Antibody Fragments. *Antibodies, 8*(2), 28. Retrieved from https://doi.org/10.3390/antib8020028

Baum, A., Ajithdoss, D., Copin, R., Zhou, A., Lanza, K., Negron, N., Ni, M., Wei, Y., Mohammadi, K., Musser, B., Atwal, G. S., Oyejide, A., Goez-Gazi, Y., Dutton, J., Clemmons, E., Staples, H. M., Bartley, C., Klaffke, B., Alfson, K., Gazi, M., … Kyratsous, C. A. (2020a). REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus macaques and hamsters. *Science, 370*(6520), 1110–1115. Retrieved from https://doi.org/10.1126/science.abe2402

Baum, A., Fulton, B. O., Wloga, E., Copin, R., Pascal, K. E., Russo, V., Giordano, S., Lanza, K., Negron, N., Ni, M., Wei, Y., Atwal, G. S., Murphy, A. J., Stahl, N., Yancopoulos, G. D., & Kyratsous, C. A. (2020b). Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science, 369*(6506), 1014–1018. Retrieved from https://doi.org/10.1126/science.abd0831

Baumann, P. (2016). Downstream process development strategies for effective bioprocesses: Trends, progress, and combinatorial approaches. *Engineering in life sciences, 17*(11), 1142–1158. Retrieved from https://doi.org/10.1002/elsc.201600033

Bestle, D. (2020). TMPRSS2 and furin are both essential for proteolytic activation of SARS-CoV-2 in human airway cells. *Life science alliance, 3*(9), e202000786. Retrieved from https://doi.org/10.26508/lsa.202000786

BioAtla. (n.d.). *Educational Appendix: Antibody Structure*. Retrieved March 2022, from BioAtla: https://www.bioatla.com/appendix/antibody-structure/

Carvalho, L. (2017). Production Processes for Monoclonal Antibodies. In A. F. Jozala, *Fermentation Processes* (p. DOI: 10.5772/64263). N.A.: IntechOpen.

Chen, B., Tian, E. K., He, B., Tian, L., Han, R., Wang, S., Xiang, Q., Zhang, S., El Arnaout, T., & Cheng, W. (2020). Overview of lethal human coronaviruses. *Sig Transduct Target Ther, 5*(89), NA. Retrieved from https://doi.org/10.1038/s41392-020-0190-2

Chevalier, A., Silva, D. A., Rocklin, G. J., Hicks, D. R., Vergara, R., Murapa, P., Bernard, S. M., Zhang, L., Lam, K. H., Yao, G., Bahl, C. D., Miyashita, S. I., Goreshnik, I., Fuller, J. T., Koday, M. T., Jenkins, C. M., Colvin, T., Carter, L., Bohn, A., Bryan, C. M., … Baker, D. (2017). Massively parallel de novo protein design for targeted therapeutics. *Nature, 550*(7674), 74–79. Retrieved from https://doi.org/10.1038/nature23912

Chiu, M. L. (2019). Antibody Structure and Function: The Basis for Engineering Therapeutics. *Antibodies, 8*(4), 55. Retrieved from https://doi.org/10.3390/antib8040055

Copin, R., Baum, A., Wloga, E., Pascal, K. E., Giordano, S., Fulton, B. O., Zhou, A., Negron, N., Lanza, K., Chan, N., Coppola, A., Chiu, J., Ni, M., Wei, Y., Atwal, G. S., Hernandez, A. R., Saotome, K., Zhou, Y., Franklin, M. C., Hooper, A. T., … Kyratsous, C. A. (2021). The monoclonal antibody combination REGEN-COV protects against SARS-CoV-2 mutational escape in preclinical and human studies. *Cell, 184*(15), 3949–3961.e11. Retrieved from https://doi.org/10.1016/j.cell.2021.06.002

Debeljak, N. F. (2006). Variability in the immunodetection of His-tagged recombinant proteins. *Analytical biochemistry, 359*(2), 216-223. Retrieved from https://doi.org/10.1016/j.ab.2006.09.017

Dou, Y., Lin, Y., Wang, T. Y., Wang, X. Y., Jia, Y. L., & Zhao, C. P. (2021). The CAG promoter maintains high-level transgene expression in HEK293 cells. *FEBS open bio, 11*(1), 95–104. Retrieved from https://doi.org/10.1002/2211-5463.13029

Eroglu, B. N. (2021). A Narrative Review of COVID-19 Vaccines. *Vaccines, 10*(1), 62. Retrieved from https://doi.org/10.3390/vaccines10010062

Esmaeilzadeh, A. R. (2021). Recent advances in antibody-based immunotherapy strategies for COVID-19. *Journal of cellular biochemistry, 122*(10), 1389–1412. Retrieved from https://doi.org/10.1002/jcb.30017

European Medicines Agency (EMA). (2022, April n.d.). *COVID-19 treatments*. Retrieved from EMA: https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/coronavirus-disease-covid-19/treatments-vaccines/covid-19-treatments

Fontana, D., Kratje, R., Etcheverrigaray, M., & Prieto, C. (2014). Rabies virus-like particles expressed in HEK293 cells. *Vaccine, 32*(24), 2799–2804. Retrieved from https://doi.org/10.1016/j.vaccine.2014.02.031

Gibaldi, M. H. (2013). Large-scale production of recombinant proteins. In M. H. Gibaldi, *Biotechnology and Biopharmaceuticals* (pp. 63-78). John Wiley & Sons, Inc. . Retrieved from https://doi.org/10.1002/9781118660485.ch5

GoldBio. (2018, May 12). *Affinity His-Tag Purification.* Retrieved from GoldBio: https://www.goldbio.com/documents/1047/Affinity%20His-Tag%20Purification%20Utilizing%20Chelating%20Agarose%20Beads.pdf

Gomes, A. (2016). An Overview of Heterologous Expression Host Systems for the Production of Recombinant Proteins. *Advances in Animal and Veterinary Sciences, 4*, 346-356. Retrieved from http://dx.doi.org/10.14737/journal.aavs/2016/4.7.346.356

Gupta, V. S. (2016). Production of Recombinant Pharmaceutical Proteins. *Basic and Applied Aspects of Biotechnology*, 77–101. Retrieved from https://doi.org/10.1007/978-981-10-0875-7_4

Han, P., Li, L., Liu, S., Wang, Q., Zhang, D., Xu, Z., Han, P., Li, X., Peng, Q., Su, C., Huang, B., Li, D., Zhang, R., Tian, M., Fu, L., Gao, Y., Zhao, X., Liu, K., Qi, J., Gao, G. F., … Wang, P. (2022). Receptor binding and complex structures of human ACE2 to spike RBD from omicron and delta SARS-CoV-2. *Cell, 185*(4), 630–640.e10. Retrieved from https://doi.org/10.1016/j.cell.2022.01.001

Hansen, J. B. (2020). Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody cocktail. *Science, 369*(6506), 1010–1014. Retrieved from https://doi.org/10.1126/science.abd0827

Ho, S. C. (2013). Control of IgG LC:HC ratio in stably transfected CHO cells and study of the impact on expression, aggregation, glycosylation and conformational stability. *Journal of biotechnology, 165*(3-4), 157–166. Retrieved from https://doi.org/10.1016/j.jbiotec.2013.03.019

Ho, S. C., Bardor, M., Feng, H., Mariati, Tong, Y. W., Song, Z., . . . Yang, Y. (2012). IRES-mediated Tricistronic vectors for enhancing generation of high monoclonal antibody expressing CHO cell lines. *Journal of biotechnology*, 130–139. doi:10.1016/j.jbiotec.2011.09.023

Hu, W., Xiang, J. Y., Kong, P., Liu, L., Xie, Q., & Xiang, H. (2017). Expression and Characterization of a Single-Chain Variable Fragment against Human LOX-1 in Escherichia coli and Brevibacillus choshinensis. *J. Microbiol. Biotechnol, 27*, 965-974. Retrieved from https://doi.org/10.4014/jmb.1702.02007

Hust, M., Jostock, T., Menzel, C., Voedisch, B., Mohr, A., Brenneis, M., . . . Dübel, S. (2007). Single chain Fab (scFab) fragment. *BMC Biotechnol*.

Ionescu, R. M. (2008). Contribution of variable domains to the stability of humanized IgG1 monoclonal antibodies. *Journal of pharmaceutical sciences, 97*(4), 1414–1426. Retrieved from https://doi.org/10.1002/jps.21104

Jacobsen, L. B. (2004). FuGENE 6 Transfection Reagent: the gentle power. *Methods, 33*(2), 104-112.

Janeway, C. J. (2001). The structure of a typical antibody molecule. In C. Janeway, *Immunobiology: The Immune System in Health and Disease. 5th edition.* (p. N.A.). New York: Garland Science.

Jespers, L., Schon, O., Famm, K., & Winter, G. (2004). Aggregation-resistant domain antibodies selected on phage by heat denaturation. *Nat Biotechnol, 22*, 1161–1165. Retrieved from https://doi.org/10.1038/nbt1000

Kang, T. H. (2020). Solubility, Stability, and Avidity of Recombinant Antibody Fragments Expressed in Microorganisms. *Frontiers in microbiology, 11*, 1927. Retrieved from https://doi.org/10.3389/fmicb.2020.01927

Kaufman, R. J. (2000). Overview of vector design for mammalian gene expression. *Molecular biotechnology, 16*(2), 151–160. Retrieved from https://doi.org/10.1385/MB:16:2:151

Khan, K. H. (2013). Gene expression in Mammalian cells and its applications. *Advanced pharmaceutical bulletin, 3*(2), 257–263. Retrieved from https://doi.org/10.5681/apb.2013.042

Kiyoshi, M., Caaveiro, J. M., Miura, E., Nagatoishi, S., Nakakido, M., Soga, S., Shirai, H., Kawabata, S., & Tsumoto, K. (2014). Affinity improvement of a therapeutic antibody by structure-based computational design: generation of electrostatic interactions in the transition state stabilizes the antibody-antigen complex. *PloS one, 9*(1), e87099. Retrieved from https://doi.org/10.1371/journal.pone.0087099

Köhler, G., & Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature, 256*, 495–497. Retrieved from https://doi.org/10.1038/256495a0

Kozak, M. (1999). Initiation of translation in prokaryotes and eukaryotes. *Gene, 234*(2), 187–208. Retrieved from https://doi.org/10.1016/s0378-1119(99)00210-3

Kyriakidis, N. L.-C. (2021). SARS-CoV-2 vaccines strategies: a comprehensive review of phase 3 candidates. *npj Vaccines, 6*, 28. Retrieved from https://doi.org/10.1038/s41541-021-00292-w

Lent, J. V. (2021). Miniaturized single-cell technologies for monoclonal antibody discovery. *Lab on a Chip, 21*(19), 3627-3654. Retrieved from https://doi.org/10.1039/D1LC00243K

Li, F., Vijayasankaran, N., Shen, A. Y., Kiss, R., & Amanullah, A. (2010). Cell Culture Processes in Monoclonal Antibody Production. *mAbs, 2*(5), 466-479. Retrieved from https://doi.org/10.4161/mabs.2.5.12720

Li, Q. N. (2021). SARS-CoV-2 501Y.V2 variants lack higher infectivity but do have immune escape. *Cell, 184*(9), 2362–2371. Retrieved from https://doi.org/10.1016/j.cell.2021.02.042

Lin, X., O'Reilly Beringhs, A., & Lu, X. (2021). Applications of Nanoparticle-Antibody Conjugates in Immunoassays and Tumor Imaging. *The AAPS Journal, 23*(2), 43. Retrieved from DOI: 10.1208/s12248-021-00561-5

Lin, Y. C. (2014). Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat Commun, 5*, 4767. Retrieved from https://doi.org/10.1038/ncomms5767

Lu, R. Z. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet, 395*(10224), 565–574. Retrieved from https://doi.org/10.1016/S0140-6736(20)30251-8

Ma, H. Ó. (2020). Antibody stability: A key to performance - Analysis, influences and improvement. *Biochimie, 177*, 213–225. Retrieved from https://doi.org/10.1016/j.biochi.2020.08.019

Majumder, J., & Minko, T. (2021). Recent Developments on Therapeutic and Diagnostic Approaches for COVID-19. *AAPS J.*, 14.

Makrides, S. C. (2003). Vectors for gene expression in mammalian cells. *New Comprehensive Biochemistry, 38*, 9–26. Retrieved from https://doi.org/10.1016/S0167-7306(03)38002-0

Mendiola-Pastrana, I. R., López-Ortiz, E., Río de la Loza-Zamora, J. G., González, J., Gómez-García, A., & López-Ortiz, G. (2022). SARS-CoV-2 Variants and Clinical Outcomes: A Systematic Review. *Life, 12*(2), 170. Retrieved from https://doi.org/10.3390/life12020170

Merck. (2022). *PSF-CAG-AMP - CAG PROMOTER PLASMID*. Retrieved from Merck: https://www.sigmaaldrich.com/FI/en/product/sigma/ogs504

Mitra, S., & Tomar, P. C. (2021). Hybridoma technology; advancements, clinical significance, and future aspects. *Journal, genetic engineering & biotechnology, 19*(1), 159. Retrieved from https://doi.org/10.1186/s43141-021-00264-6

Muhammed, Y. (2020). The Best IgG Subclass for the Development of Therapeutic Monoclonal Antibody Drugs and their Commercial Production: A Review. *Immunome Research, 16*(1), 173.

Neerukonda, S. V. (2021). Establishment of a well-characterized SARS-CoV-2 lentiviral pseudovirus neutralization assay using 293T cells with stable expression of ACE2 and TMPRSS2. *PLoS ONE, 16*(3). Retrieved from https://doi.org/10.1371/journal.pone.0248348

New England Biolabs Inc. (n.d., n.d. n.d.). *Products: Restriction Endonucleases*. Retrieved March 2022, from New England Biolabs Inc.: https://international.neb.com/products/r3101-ecori-hf

Pang, N. P. (2021). Understanding neutralising antibodies against SARS-CoV-2 and their implications in clinical practice. *Military Med Res, 8*, 47. Retrieved from https://doi.org/10.1186/s40779-021-00342-3

Parra-Lucares, A. S.-P. (2022). Emergence of SARS-CoV-2 Variants in the World: How Could This Happen? *Life, 12*(2), 194. Retrieved from https://doi.org/10.3390/life12020194

Polack, F. P. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *The New England journal of medicine, 383*(26), 2603–2615. Retrieved from https://doi.org/10.1056/NEJMoa2034577

Pomplun, S. (2021). Targeting the SARS-CoV-2-spike protein: from antibodies to miniproteins and peptides. *RSC Medicinal Chemistry*(2), 197-202. Retrieved from https://doi.org/10.1039/D0MD00385A

Prassler, J., & Stark, Y. (2004). *US Patent No. WO 2004/013276 A2.* Retrieved from https://patentimages.storage.googleapis.com/ca/1a/d1/aef38db283fb2b/WO2004013276A2.pdf

Promega. (2011). *Technical Manual. Renilla-Glo Luciferase Assay System.* Retrieved from Promega: https://worldwide.promega.com/-/media/files/resources/protocols/technical-manuals/101/renilla-glo-luciferase-assay-system-protocol.pdf?rev=66a92baf991449cab976e8ad24a1987c&sc_lang=en

Raghuvamsi, P. V., Tulsian, N. K., Samsudin, F., Qian, X., Purushotorman, K., Yue, G., Kozma, M. M., Hwa, W. Y., Lescar, J., Bond, P. J., MacAry, P. A., & Anand, G. S. (2021). SARS-CoV-2 S protein:ACE2 interaction reveals novel allosteric targets. *eLife, 10*, e63646. Retrieved from https://doi.org/10.7554/eLife.63646

Regeneron Pharmaceuticals. (2022). Retrieved February 14, 2022, from https://www.regeneron.com/covid19

Rnjak, D. R. (2021). COVID-19 convalescent plasma as long-term therapy in immunodeficient patients? *Transfusion clinique et biologique : journal de la Societe francaise de transfusion sanguine, 28*(3), 264–270. Retrieved from https://doi.org/10.1016/j.tracli.2021.04.004

Robinson, P. C. (2022). COVID-19 therapeutics: Challenges and directions for the future. *Proceedings of the National Academy of Sciences of the United States of America, 119*(15), e2119893119. Retrieved from https://doi.org/10.1073/pnas.2119893119

Roth, D. B. (2014). V(D)J Recombination: Mechanism, Errors, and Fidelity. *Microbiology spectrum, 2*(6), 10.1128/microbiolspec.MDNA3-0041-2014. Retrieved from https://doi.org/10.1128/microbiolspec.MDNA3-0041-2014

Roth, K. W. (2021). Developing Recombinant Antibodies by Phage Display Against Infectious Diseases and Toxins for Diagnostics and Therapy. *Frontiers in cellular and infection microbiology, 11*, 697876. Retrieved from https://doi.org/10.3389/fcimb.2021.697876

Santos-Sánchez, N. F., & Salas-Coronado, R. (2020). Origin, structural characteristics, prevention measures, diagnosis and potential drugs to prevent and COVID-19. *Medwave, 20*(8), e8037. Retrieved from https://doi.org/10.5867/medwave.2020.08.8037

Santos, M. L. (2018). Advances and challenges in therapeutic monoclonal antibodies drug development. *Brazilian Journal of Pharmaceutical Sciences, 54*, e01007. Retrieved from https://doi.org/10.1590/s2175-97902018000001007

Satheeshkumar, P. (2020). Expression of Single Chain Variable Fragment (scFv) Molecules in Plants: A Comprehensive Update. *Mol Biotechnol, 62*, 151–167. Retrieved from https://doi.org/10.1007/s12033-020-00241-3

Schlatter, S. S. (2005). On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. *Biotechnology progress, 21*(1), 122–133. Retrieved from https://doi.org/10.1021/bp049780w

Schoeman, D. G. (2021). Pathogenic Human Coronaviruses. *Reference Module in Biomedical Sciences*, B978-0-12-818731-9.00052-5. Retrieved from https://doi.org/10.1016/B978-0-12-818731-9.00052-5

Sidhu, S. S. (2001). Engineering M13 for phage display. *Biomolecular engineering, 18*(2), 57-63. Retrieved from https://doi.org/10.1016/s1389-0344(01)00087-9

Sifniotis, V. C. (2019). Current Advancements in Addressing Key Challenges of Therapeutic Antibody Design, Manufacture, and Formulation. *Antibodies, 8*(2), 36. Retrieved from https://doi.org/10.3390/antib8020036

Siracusano, G., Brombin, C., Pastori, C., Cugnata, F., Noviello, M., Tassi, E., Princi, D., Cantoni, D., Malnati, M. S., Maugeri, N., Bozzi, C., Saretto, G., Clementi, N., Mancini, N., Uberti-Foppa, C., Temperton, N., Bonini, C., Di Serio, C., & Lopalco, L. (2021). Profiling Antibody Response Patterns in COVID-19: Spike S1-Reactive IgA Signature in the Evolution of SARS-CoV-2 Infection. *Frontiers in immunology, 12*, 772239. Retrieved from https://doi.org/10.3389/fimmu.2021.772239

Steward, K. (2021, March 24). *Pandemic, Leveraging Pseudoviruses in the Face of the COVID-19*. Retrieved from https://www.technologynetworks.com/immunology/articles/leveraging-pseudoviruses-in-the-face-of-the-covid-19-pandemic-347021

Tan, E. C. (2021). HEK293 Cell Line as a Platform to Produce Recombinant Proteins and Viral Vectors. *Frontiers in bioengineering and biotechnology, 9*, 796991. Retrieved from https://doi.org/10.3389/fbioe.2021.796991

Tiller, T. M. (2008). Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *Journal of immunological methods, 329*(1-2), 112–124. Retrieved from https://doi.org/10.1016/j.jim.2007.09.017

Tripathi, N. K. (2019). Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. *Frontiers in bioengineering and biotechnology, 7*, 420. Retrieved from https://doi.org/10.3389/fbioe.2019.00420

Tschorn, N. B. (2020). Transposon vector-mediated stable gene transfer for the accelerated establishment of recombinant mammalian cell pools allowing for high-yield production of biologics. *Biotechnol Lett, 42*, 1103–1112. Retrieved from https://doi.org/10.1007/s10529-020-02889-y

US Biological. (2022). *II), N2008-05 Rabbit Anti-Neomycin Phosphotransferase II (NPT*. Retrieved from US Biological: https://www.usbio.net/antibodies/N2008-05

US FDA Administration. (2022, April 11). *COVID-19 EUAs: Drugs and Non-Vaccine Biological Products*. Retrieved from FDA: https://www.fda.gov/emergency-preparedness-and-response/mcm-legal-regulatory-and-policy-framework/emergency-use-authorization#coviddrugs

US FDA Administration. (2022, January 24). *FDA STATEMENT Coronavirus (COVID-19) Update: FDA Limits Use of Certain Monoclonal Antibodies to Treat COVID-19 Due to the Omicron Variant*. Retrieved from FDA: https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-limits-use-certain-monoclonal-antibodies-treat-covid-19-due-omicron

Ventouri, I. K. (2020). Probing Protein Denaturation during Size-Exclusion Chromatography Using Native Mass Spectrometry. *Analytical chemistry*, 4292–4300. Retrieved from https://doi.org/10.1021/acs.analchem.9b04961

Wang, S. L. (2022, February). Establishment of a pseudovirus neutralization assay based on SARS-CoV-2 S protein incorporated into lentiviral particles. *Biosafety*

and Health, 4(1), 38-44. Retrieved from https://doi.org/10.1016/j.bsheal.2021.12.006

Weinreich, D. M., Sivapalasingam, S., Norton, T., Ali, S., Gao, H., Bhore, R., Musser, B. J., Soo, Y., Rofail, D., Im, J., Perry, C., Pan, C., Hosain, R., Mahmood, A., Davis, J. D., Turner, K. C., Hooper, A. T., Hamilton, J. D., Baum, A., Kyratsous, C. A., … Trial Investigators (2021a). REGN-COV2, a Neutralizing Antibody Cocktail, in Outpatients with Covid-19. *N Engl J Med, 384*(3), 238-251.

Weinreich, D. M. (2021b). REGEN-COV Antibody Combination and Outcomes in Outpatients with Covid-19. *The New England journal of medicine, 385*(23), e81. Retrieved from https://doi.org/10.1056/NEJMoa2108163

World Health Organization. (2020, January 20). *Novel Coronavirus (2019-nCoV) Situation report, 1.* Retrieved from World Health Organization: https://apps.who.int/iris/handle/10665/330760

World Health Organization. (2020). *Novel Coronavirus (2019-nCoV): situation report, 1.* Iris.

World Health Organization. (2022, April 6). *Tracking SARS-CoV-2 Variants*. Retrieved April 2022, from World Health Organization: https://www.who.int/en/activities/tracking-SARS-CoV-2-variants

Wu, C. R., Yin, W. C., Jiang, Y., & Xu, H. E. (2022). Structure genomics of SARS-CoV-2 and its Omicron variant: drug design templates for COVID-19. *Acta pharmacologica Sinica*, 1–13. Retrieved from https://doi.org/10.1038/s41401-021-00851-w

Wu, S. C., Meir, Y. J., Coates, C. J., Handler, A. M., Pelczar, P., Moisyadi, S., & Kaminski, J. M. (2006). piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. *Proceeding of the National Academy of Sciences of the United States of America, 103*(41), 15008-15013. Retrieved from https://doi.org/10.1073/pnas.0606979103

Xiao, K. Y. (2021). Antibodies Can Last for More Than 1 Year After SARS-CoV-2 Infection: A Follow-Up Study From Survivors of COVID-19. *Frontiers in medicine, 8*, 684864. Retrieved from https://doi.org/10.3389/fmed.2021.684864

Yang, H., & Rao, Z. (2021). Structural biology of SARS-CoV-2 and implications for therapeutic development. *Nature reviews. Microbiology, 19*(11), 685–700. Retrieved from https://doi.org/10.1038/s41579-021-00630-8

Yeo, J. M. (2018). An IRES-Mediated Tricistronic Vector for Efficient Generation of Stable, High-Level Monoclonal Antibody Producing CHO DG44 Cell Lines. *Methods in molecular biology, 1827*, 335-349.

Yeo, J., Mariati, & Yang, Y. (2018). An IRES-Mediated Tricistronic Vector for Efficient Generation of Stable, High-Level Monoclonal Antibody Producing CHO DG44 Cell Lines. *Methods in molecular biology (Clifton, N.J.)*, 335–349. doi:10.1007/978-1-4939-8648-4_17

Zervou, F. N., Louie, P., Stachel, A., Zacharioudakis, I. M., Ortiz-Mendez, Y., Thomas, K., & Aguero-Rosenfeld, M. E. (2021). SARS-CoV-2 antibodies: IgA correlates with severity of disease in early COVID-19 infection. *Journal of medical virology, 93*(9), 5. Retrieved from https://doi.org/10.1002/jmv.27058

Zheng, K. B. (2011). The impact of glycosylation on monoclonal antibody conformation and stability. *mAbs, 3*(6), 568–576. Retrieved from https://doi.org/10.4161/mabs.3.6.17922

Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G. F., Tan, W., & China Novel Coronavirus Investigating and Research Team (2020a). A Novel Coronavirus from Patients with Pneumonia in China, 2019. *The New England journal of medicine, 382*(8), 727–733. Retrieved from https://doi.org/10.1056/NEJMoa2001017

Zhu, Z., Lian, X., Su, X., Wu, W., Marraro, G. A., & Zeng, Y. (2020b). From SARS and MERS to COVID-19: a brief summary and comparison of severe acute respiratory infections caused by three highly pathogenic human coronaviruses. *Respir Res, 21*, 224. Retrieved from https://doi.org/10.1186/s12931-020-01479-w

APPENDICES

Appendix 1. *LIH1 DNA sequence.*

REGN10987 antibody Fab fragment light chain [Homo sapiens]
IRES
REGN10987 antibody Fab fragment heavy chain (without Fc part) [Homo sapiens]

```
GATATCTGATCAGCTCAGAATTCACTAGTGCCGCCACCATGAAGTGGGTCACCTTTATTAGCCTCTT
GTTTCTCTTCTCAAGTGCATACAGCCAGTCAGCTCTTACCCAACCTGCTTCCGTTTCCGGAAGTCCA
GGCCAATCCATAACTATAAGCTGCACAGGCACTTCCAGCGATGTTGGCGGGTATAACTACGTTAGCT
GGTACCAACAACATCCAGGTAAAGCTCCCAAGTTGATGATTTACGATGTTTCAAAGAGGCCCAGTGG
TGTTAGTAATAGGTTTAGTGGGTCAAAGTCAGGTAATACAGCATCTCTCACCATTAGTGGTCTGCAA
TCCGAGGACGAAGCCGACTACTACTGCAATAGTTTGACGTCTATCTCAACGTGGGTGTTTGGTGGTG
GGACGAAACTCACTGTCCTCGGCCAACCTAAAGCTGCACCTAGTGTTACCCTCTTTCCCCCATCATC
CGAAGAATTGCAAGCAAACAAAGCAACACTCGTGTGTCTGATCTCCGACTTCTACCCCGGTGCTGTG
ACAGTCGCATGGAAGGCTGATTCCTCTCCAGTGAAAGCCGGCGTCGAAACAACCACCCCTAGTAAAC
AATCTAACAACAAATATGCAGCCTCATCATATTTGTCACTTACCCCTGAGCAATGGAAAAGCCACCG
ATCTTACAGCTGTCAAGTGACTCACGAAGGTTCCACAGTCGAAAAGACCGTTGCTCCCACAGAGTGT
TCTTGAAGATCTCCCCTCTCCCTCCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGC
CGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGA
AACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGG
TCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCG
ACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTAT
AAGATACACCTGCAAAGGCGGCACAACCCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGT
CAAATGGCTCTCCTCAAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATG
GGATCTGATCTGGGGCCTCGGTACACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAG
GCCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACAACCATGC
CGCTTCTCCTACTGCTGCCACTTCTATGGGCCGGCGCGCTAGCACAAGTACAGCTGGTCGAGTCCGG
TGGCGGCGTGGTGCAACCAGGACGCTCCTTGCGGCTGTCATGCGCTGCTTCCGGGTTCACATTTTCC
AATTACGCTATGTATTGGGTTCGGCAAGCCCCTGGTAAAGGCCTGGAATGGGTGGCTGTTATATCTT
ATGACGGAAGCAACAAGTATTACGCAGATAGTGTGAAGGGGCGTTTTACAATTTCTCGCGATAACTC
CAAGAATACTCTCTATTTGCAAATGAACAGTCTAAGGACAGAAGATACAGCTGTCTATTATTGCGCT
AGTGGAAGCGATTACGGTGATTATCTCCTTGTATACTGGGGTCAAGGAACTCTGGTAACGGTGAGCT
CCGCCAGCACTAAAGGCCCAAGTGTTTTCCCCCTGGCTCCGTCTTCTAAATCAACTTCTGGTGGTAC
CGCAGCACTCGGATGTCTGGTAAAGGATTATTTCCCCGAGCCAGTGACCGTCTCTTGGAACTCCGGC
GCCCTAACATCCGGGGTTCATACCTTCCCAGCTGTCCTGCAAAGCTCTGGCTTGTATTCCCTGTCCT
CTGTTGTAACTGTTCCTAGCAGCTCCCTTGGCACCCAGACCTATATTTGTAACGTAAATCACAAACC
TTCCAACACCAAAGTAGATAAGAAAGTTGAGCCCAAGTCCTGTGATAAAACACATGGCGGCTCAGGC
GGTTCAGGTGGATCCCATCACCACCATCACCACCATCATTGAGCGGCCGCACGCGTTCTAGAGATAT
C
```

Appendix 2. *LIH2 DNA sequence.*

REGN10933 antibody Fab fragment light chain [Homo sapiens]
IRES
REGN10933 antibody Fab fragment heavy chain (without Fc part) [Homo sapiens]

```
GATATCTGATCAGCTCAGAATTCACTAGTGCCGCCACCATGCCTCTGCTACTTCTGCTCCCACT
CCTATGGGCTGGCGCCCTTGCCGACATTCAGATGACACAATCCCCCAGCTCTCTAAGTGCTTCT
GTAGGGGACCGCGTCACAATTACATGTCAAGCCAGTCAAGACATCACCAATTACCTGAATTGGT
ACCAGCAAAAGCCCGGGAAGGCCCCAAAGCTTCTCATCTACGCTGCCAGTAATCTTGAAACCGG
CGTTCCCTCTAGGTTTTCAGGTAGCGGCAGCGGAACCGATTTTACGTTCACAATCTCTGGGCTG
CAGCCAGAGGACATCGCAACCTATTACTGCCAACAATACGACAATTTGCCTCTGACTTTCGGAG
GAGGTACCAAAGTAGAAATCAAAAGGACCGTTGCCGCACCAAGCGTCTTTATTTTCCCGCCCTC
TGATGAGCAACTAAAATCTGGCACCGCATCCGTGGTCTGTTTGTTGAACAACTTTTACCCACGA
GAGGCAAAAGTCCAGTGGAAAGTTGACAATGCTCTCCAATCAGGCAATTCACAGGAATCCGTAA
CTGAACAAGATTCAAAAGACTCCACATATTCCTTGAGTAGTACACTGACCCTCAGCAAAGCAGA
CTACGAAAAGCATAAGGTGTACGCTTGCGAGGTGACACATCAAGGGCTCTCATCACCAGTTACC
AAGAGCTTCAATCGCGGAGAATGTTGAAGATCTCCCCTCTCCCTCCCCCCCCCCTAACGTTACT
GGCCGAAGCCGCTTGGAATAAGGCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGC
CGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGG
TCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTG
GAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTG
GCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACC
CCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAGCGTATTC
AACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCTGGGCCTCGGT
ACACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGA
CGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCACAACCATGCCGCTTCTCCTACTGCT
GCCACTTCTATGGGCCGGCGCGCTAGCACAGGTGCAACTGGTGGAGAGTGGCGGTGGGCTGGTA
AAACCAGGCGGCAGCCTTCGACTTAGCTGCGCCGCTAGTGGGTTCACTTTTAGCGATTACTACA
TGAGCTGGATTCGCCAAGCTCCTGGCAAAGGTCTCGAATGGGTTTCCTACATAACATATTCTGG
TTCAACGATTTACTATGCTGATAGTGTTAAGGGCAGATTTACTATAAGCCGCGACAACGCCAAG
TCTTCCCTCTATCTCCAAATGAATAGCCTGCGTGCTGAAGATACAGCAGTGTACTACTGCGCAC
GGGATAGAGGGACCACCATGGTGCCCTTCGACTATTGGGGCCAAGGCACACTTGTCACAGTTAG
CAGTGCTTCTACTAAGGGTCCTTCCGTATTTCCTCTTGCCCCATCTTCTAAATCTACATCCGGT
GGGACCGCAGCCCTCGGCTGCTTGGTGAAAGATTACTTCCCGGAGCCAGTGACTGTGTCCTGGA
ATAGCGGAGCCCTAACCAGTGGAGTACACACTTTCCCCGCCGTTCTACAATCTAGTGGGCTGTA
TAGTTTGTCTTCCGTCGTTACAGTCCCCAGCTCCAGCCTGGGCACCCAAACTTATATCTGCAAC
GTTAATCATAAACCCTCAAACACAAAAGTCGACAAGAAGGTGGAGCCAAAGTCTTGTGATAAAA
CACATGGTGGCTCTGGTGGTTCAGGCGGATCCCACCATCATCACCACCACCATCATTGAGCGGC
CGCACGCGTTCTAGAGATATC
```

Appendix 3. *SCF1 DNA sequence.*

REGN10987 antibody derived scFv VLC-linker-VHC-linker-BamHI-8his

```
GATATCTGATCAGCTCAGAATTCGCTAGCGCCGCCACCATGAAATGGGTGACTTTCATATCCCT
GCTCTTCTTGTTCTCTTCAGCCTATTCCCAAAGTGCACTGACACAGCCTGCCAGCGTCAGTGGA
AGTCCAGGCCAATCCATTACAATCAGCTGTACAGGAACATCTAGTGACGTGGGCGGTTACAATT
ATGTAAGCTGGTATCAACAACATCCAGGTAAAGCTCCCAAGCTAATGATCTATGATGTAAGTAA
GAGACCTTCCGGGGTCTCCAATCGGTTCAGTGGATCAAAATCCGGCAATACAGCCAGCCTAACC
ATTAGTGGACTCCAAAGCGAAGACGAAGCCGATTATTATTGTAATTCACTGACTAGCATTAGCA
CTTGGGTCTTTGGGGGGGGAACTAAGTTGACCGTACTGGGACAAGGTGGGGGGGGAAGTGGCGC
TGGCGGCTCAGGTGGCGGCGGAACAGGCGGTGGTGGCAGCCAAGTGCAGTTGGTCGAAAGCGGT
GGCGGAGTGGTGCAGCCTGGCCGCTCCCTGCGGCTGAGTTGCGCAGCTTCCGGGTTCACCTTTA
GCAATTATGCCATGTACTGGGTAAGACAAGCACCGGGGAAAGGACTAGAATGGGTCGCCGTTAT
CTCCTACGACGGTTCTAACAAATATTACGCCGACAGTGTCAAGGGCAGGTTTACAATATCTCGC
GATAATTCCAAGAATACCTTGTATCTCCAAATGAACTCTCTGAGAACTGAGGATACTGCCGTCT
ATTATTGCGCAAGCGGCAGCGACTACGGGGACTATCTGCTTGTGTATTGGGGCCAAGGCACTCT
TGTCACTGTGTCTTCCGGCGGAGGAGGTAGTGGCGGCGGCGGTTCCGGTGGCGGCGGCTCTGGC
GGCGGCGGCTCAGGTGGTGGTGGATCCCACCATCATCACCACCACCATCATTGAGCGGCCGCAC
GCGTTCTAGAGATATC
```

Appendix 4. *SCF2 DNA sequence.*

REGN10933 antibody derived scFv VLC-linker-VHC-linker-BamHI-8his

```
GATATCTGATCAGCTCAGAATTCGCTAGCGCCGCCACCATGCCACTTTTGTTGTTGCTACCACT
CCTCTGGGCTGGCGCTCTCGCAGATATTCAAATGACACAATCACCGTCTAGTCTTTCCGCATCT
GTCGGCGATAGAGTTACCATCACATGCCAAGCCTCTCAAGACATAACAAATTACCTCAATTGGT
ATCAGCAGAAACCCGGAAAAGCACCGAAGCTGCTCATATATGCAGCAAGCAATCTGGAGACTGG
GGTCCCCTCTCGATTCTCTGGAAGCGGTTCAGGTACCGACTTCACCTTTACCATCAGTGGTCTA
CAACCAGAGGATATAGCAACATATTATTGTCAACAGTATGACAACTTGCCCTTGACCTTCGGCG
GCGGAACAAAGGTCGAGATTAAAGGACAGGCGGAGGAGGAAGTGGGGCTGGTGGGTCTGGTGG
GGGGGGCACAGGTGGCGGCGGTTCACAAGTTCAACTAGTTGAAAGCGGTGGCGGGTTGGTGAAA
CCTGGTGGGAGCCTGCGTCTGAGTTGCGCCGCTTCCGGCTTCACATTTTCTGATTATTACATGA
GCTGGATTCGGCAAGCTCCCGGTAAGGGTCTCGAGTGGGTCAGTTACATCACCTACTCCGGCAG
CACGATTTACTATGCCGACTCTGTCAAGGGGCGGTTTACTATCAGCCGGGATAACGCCAAATCC
AGCCTCTACTTGCAGATGAACTCTCTTCGGGCTGAAGATACCGCAGTCTACTATTGTGCAAGAG
ATCGGGGAACCACCATGGTACCTTTTGATTACTGGGGCCAAGGGACACTGGTGACTGTTTCTAG
TGGCGGTGGTGGGAGCGGTGGTGGTGGTAGCGGCGGTGGCGGATCAGGTGGGGGGGGGATCAGGA
GGAGGTGGATCCCACCATCATCACCACCACCATCATTGAGCGGCCGCACGCGTTCTAGAGATAT
C
```

Appendix 5. *hFc (IgG2) amino acid sequence.*

```
GAGLERKSSVECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYV
DGVEVHNAKTKPREEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKGQP
REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLY
SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
```

Appendix 6. *mFc (IgG2a)-6His amino acid sequence.*

```
GSGGGGPRGPTIKPCPPCKCPAPNLLGGPSVFIFPPKIKDVLMISLSPIVTCVVVDVSEDDPDV
QISWFVNNVEVHTAQTQTHREDYNSTLRVVSALPIQHQDWMSGKEFKCKVNNKDLPAPIERTIS
KPKGSVRAPQVYVLPPPEEEMTKKQVTLTCMVTDFMPEDIYVEWTNNGKTELNYKNTEPVLDSD
GSYFMYSKLRVEKKNWVERNSYSCSVVHEGLHNHHTTKSFSRTPGKGSHHHHHH
```

Appendix 7. *Construct maps of LIH1, LIH2, SCF1, SCF2 in pUC57-simple.*

Appendix 8. Example of test restriction digestion protocol (same for all RE).

	Amount
DNA	1 µl
RE1 (e.g. EcoRI)	0,2 µl
RE2 (e.g. BamHI)	0,2 µl
CutSmart Buffer (x10)	1 µl
Ster. MQ H_2O	7,6 µl
Total	**10 µl**

+37°C 40 min or o/n

Appendix 9. Example of cloning RE digestion protocol.

	Amount	Example of SCF1 cloning digestion
DNA	5 µg	9 µl (DNA connectration 552 ng/µl)
EcoRI	1 µl	1 µl
BamHI	1 µl	1 µl
CutSmart Buffer (x10) (NEB2 for BglII)	1/10 of total X µl	1,5 µl
Ster. MQ H_2O	Fill until total X µl	2,5 µl
Total	**X µl**	**15** µl

+37°C o/n

Appendix 10. Transfection protocol.

	Amount		
DNA	1,2 µg		15 min incubation
hypPBase	0,12 µg		
Fugene 6	6 µl	5 min incubation	
DMEM 0%	120 µl		

HEK293F monolayer cells (6-well plate)

Appendix 11. *Example of IMAC protocol.*

LIH1-His8 purification

Material**:** 150 ml HEK293F in OptiCHO production media, 5 days in suspension at 33°C
Add   1M NaCl   8.7 g solid
         5 mM imidazole   0.75 ml 1M
Filter through 0.22 µm
Column: Ni-NTA Protino Macherey-Nagel 1 ml, new column. Take "in" sample.

1. Rinse with (10 ml MQ, add NiSO4,) rinse 10 ml MQ
2. Prepare sample, prepare pump. Start pumping sample at 1 ml/min, make sure no air bubbles are trapped in the pump. Pump circulating o/n.
3. Wash 1, 5 mM imidazole, 10 ml, 1 ml/min
4. Wash 2, 20 imidazole, 10 ml
5. Elution 1, 250 imidazole, 1 ml,
6. Elution 2, 250 imidazole, 1 ml,
7. Elution 3, 250 imidazole, 1 ml,
8. Elution 4, 250 imidazole, 1 ml,
9. Elution 5, 250 imidazole, 1 ml
10. Elution 6, 250 imidazole, 1 ml
11. Rinse with MQ, 10 ml
12. Store in 30 % EtOH, 10 ml

Appendix 12. *Purification protocol of LIH1-hFc via RBD-mFc-6His.*

HEK293F RBD-mFc-6His   50 ml
HEK293F LIH1-hFc   50 ml
0.5 M NaCl   5.8 g solid
5 mM imidazole   0.5 ml 1M
Filter through 0.22 µm
Column: Ni-NTA Protino Macherey-Nagel 1 ml. Take "in" sample. Pump circulating 1 ml/min o/n.
All washes and elutions contain 0.2 M NaCl, 0.02 M NaHPO4, pH 7.2

1. Wash 1, 5 mM imidazole, 10 ml, 1 ml/min
2. Wash 2, 20 mM imidazole, 5 ml
3. Elution 1-5, 20 imidazole, 7M Urea, 1M NaCl, 1 ml, 1 ml/min
4. Wash 3, 20 mM imidazole, 5 ml, 1 ml/min
5. Wash (RBD-mFc-6His) 4-7, 250 mM imidazole, 1 ml
6. MQ, 10 ml
7. EtOH 30%, 5 ml

Appendix 13. *Protein A purification protocol.*

LIH1-hFc purification

Material**:** 50 ml HEK293F in OptiCHO production media, 5 days in suspension at 33°C
No additions
Filter through 0.22 µm
Column: Protein A Sepharose (GE) 1 ml, new column. Take "in" sample.

1. Rinse with 10 ml MQ
2. Prepare sample, prepare pump. Start pumping sample at 1 ml/min, make sure no air bubbles are trapped in the pump. Pump circulating o/n.
3. Wash 1, 20 mM NaHPO4, 10 ml
4. Elutions 1-8 0.1M citric acid pH4 1 ml + 1M Tris-HCl pH9 100 µl (put in the tubes before elutions)
5. Rinse with MQ, 20 ml
6. Store in 20 % EtOH, 10 ml