

SOMAmer Technology to Diagnose Coronavirus Infection: Veterinary and Zoonotic Implications



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Declaration

This thesis is submitted to the University of Oxford in support of my application for the degree of Doctor of Philosophy. It has been composed by me and has not been submitted in any previous application for any degree. The work presented was carried out by me, including experimental design, data acquisition, data analysis, and text.

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Abbreviations

ACE	Angiotensin converting enzyme 2
AI	Artificial intelligence
AIDS	Acquired Immunodeficiency Syndrome
APS	Ammonium persulphate
ASSURED	Affordable, sensitive, specific, user-friendly, rapid and robust
AU	Gold
BCoV	Bovine coronavirus
BRIDGE	Biosecurity Readiness through Intelligence, Data and Global Engagement
BSA	Bovine serum albumin
CCoV	Canine coronavirus
CEPI	Coalition for Epidemic Preparedness Innovation
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
Ct	Cycle Threshold
CTD	C-terminal domain
DARP	Designed ankyrin repeat proteins
DEFRA	Department of the Environment Food and Rural Affairs
DM	Day Mission
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DPP	Dipeptidyl Peptidase
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide acid assay
EM	Electron microscopy
ERGIC	Endoplasmic reticulum–Golgi intermediate compartment
FCoV	Feline coronavirus
FECV	Feline enteric coronavirus
FIND	Foundation for Innovative New Diagnostics
FIPV	Feline infectious peritonitis virus
FL	Full length
FMD	Foot and Mouth Disease
FMDV	Foot and Mouth Disease Virus
GDP	Gross domestic product
HCoV	Human coronavirus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIS	Histidine
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IBV	Infectious bronchitis virus

ICTV	International Committee on Taxonomy of Viruses
IHC	Immunohistochemistry
IPPS	International Pandemic Preparedness Secretariat
LAMP	Loop-mediated isothermal amplification
LFA	Lateral flow immunoassays
LFD	Lateral flow devices
LFT	Lateral flow test
LLOD	Lower limit of detection
LOD	Limit of detection
MEM	Minimum Essential Medium
MERS	Middle Eastern Respiratory Syndrome
mNGS	Metagenomic next generation sequencing
MRE	Molecular recognition element
MW	Molecular weight
NAAT	Nucleic acid amplification tests
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NP	Nanoparticle
NPV	Negative predictive value
NSP	Non-structural protein
NTD	N-terminal domain
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PANGO	Phylogenetic Assignment of Named Global Outbreak
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCoV	Porcine deltacoronavirus
PEDV	Porcine epidemic diarrhoea virus
PFU	Plaque forming units
PHEV	Porcine hemagglutinating encephalomyelitis virus
PPV	Positive predictive value
PRCV	Porcine respiratory coronavirus
RBD	Receptor binding domain
RBM	Receptor binding motif
REASSURED	Real-time connectivity, affordable, sensitive, specific, user-friendly, rapid and robust
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT	Reverse transcription
RTC	Replication-transcription complex
RU	Response units
SADS-CoV	Swine acute diarrhoea syndrome coronavirus
SARS	Severe Acute Respiratory Syndrome

SDS	Sodium dodecyl sulphate
SELEX	Systematic evolution of ligands by exponential enrichment
SENPAH	PAH (Polyallylamine hydrochloride)-modified aptamer
SOC	Super Optimal broth with Catabolite repression
SOMAmer	Slow off rate modified aptamer
SPR	Surface plasmon resonance
SS	Single stranded
TBE	Tris-borate-EDTA
TEMED	Tetramethylethylenediamine
TGEV	Transmissible gastroenteritis virus
TMB	3, 3', 5, 5'-Tetramethylbenzidine
TPP	Target product profile
UK	United Kingdom
UKHSA	United Kingdom Health Security Agency
URL	Uniform resource locator
UV	Ultraviolet
VOC	Variant of concern
VOI	Variant of interest
WHO	World Health Organisation
WOAH	World Organisation for Animal Health

Abstract

The severe effects of the COVID-19 pandemic on human health, healthcare systems, and its social and economic impacts led to the development of the 100 Days Mission. This initiative has the ambition to respond to infectious disease threats within 100 days of recognition of a potential pandemic to ensure the supply of diagnostics, vaccines and therapeutics. A key requirement for the rapid, scalable, and affordable production of diagnostic tools is the development of molecular recognition elements (MREs) which for antigen detection are currently based on monoclonal antibodies (mAbs). Compared with mAbs, ssDNA SOMAmers offer a potential alternative for a more rapid and cost-effective solution to the 100DM challenge. Research was undertaken to evaluate a library of SOMAmers raised in 2020 against the SARS-CoV-2 Wuhan-Hu-1 strain spike protein and its subdomains. An ELONA assay was developed and used to determine the binding properties of SOMAmers with a broad range of coronaviruses, including SARS-CoV-2 variants, sarbecoviruses, and viruses from the wider alpha, beta, and gammacoronavirus genera. Individual SOMAmers displayed varying binding patterns, either species-specific against SARS-CoV-2 or across sarbecoviruses, but with limited binding responses against more diverse genera. Selected SOMAmers were evaluated using competition-based approaches to identify non-competing epitope pairs that function as capture and detection MREs. SOMAmer pairs were used in the early development of a lateral flow test as a rapid, scalable, portable mass-testing platform for the detection of viral antigens as a screening or confirmatory diagnostic. An early prototype LFT based exclusively on SOMAmers was demonstrated, and further optimisation and development would be required before full validation and field trials.

Through these studies, we deepened our understanding of the potential for SOMAmers to replace mAbs in a rapid antigen diagnostic test, further work is required to determine whether SOMAmers can meet the challenges posed by the 100DM aspirations.

Chapter 1: Introduction

1.1 The pandemic challenge and role of diagnostics

1.1.1 The pandemic challenge and preparation

Over human history, infectious disease has caused major challenges to human survival and progress in parallel with war and famine. For example, the Black Death caused by *Yersinia pestis* [1] smallpox [2] and influenza [3] pandemics were responsible for millions of deaths and the reshaping of human society. In the modern era, infectious diseases such as HIV/AIDS [4] and COVID-19 [5] have reminded us that vigilance is still required as the risk of worldwide impacts persists. Science and medicine must continue to prepare and innovate to respond to these threats.

The severe effects of the COVID-19 pandemic on human health, healthcare systems, and its social and economic impacts led to the development of the 100 Days Mission [6]. This initiative was launched by the G7 in 2021 with the ambition to respond to infectious disease threats within 100 days of recognition of a potential pandemic. The International Pandemic Preparedness Secretariat [7] co-ordinates and reports on progress, highlighting continuing gaps and challenges.

In the UK, a cross-government group published a framework to enable the UK's focused research and development approach in preparation for, and during, a pandemic [8]. This framework also encompassed lessons from the Chief Medical Officer's technical report on the COVID-19 pandemic and supports requirements of the UK Biological Security Strategy. In preparation for a pandemic or major epidemic, the aims of the framework are to support research to (a) understand the pathogen and disease, (b) improve information for policy and clinical decision-making, (c) develop and improve tools and treatments to prevent and mitigate its effects and (d) ensure

strong existing research infrastructure and capacity (with appropriate funding) that can pivot in an emergency.

Understandably, a key focus of the 100DM is to ensure the supply of diagnostics, vaccines and therapeutics within 100 days of identification of a pandemic threat. As also recognised in the UK R&D framework, this trio of tools serve to detect, prevent, and treat infections caused by pathogens with pandemic potential, thereby limiting pathology, transmission, and case fatality.

Diagnostic tools are critical in identifying and confirming infection to enable treatment and isolation to reduce transmission. The 100-day timeline reflects a brief window where actions can determine whether an outbreak is contained or may escalate into a pandemic. To meet the 100DM **A**ffordable, **S**ensitive, **S**pecific, **U**ser-friendly, **R**apid and robust, **E**quipment-free, and **D**eliverable (ASSURED) diagnostic tools need to be developed and manufactured at scale rapidly [9].

1.1.2 The pandemic risk from zoonoses

Many recent pandemics have originated from zoonotic transmission events, in which animal viruses crossed species barriers into human populations [10]. Consequently, understanding viral diversity, ecology, and abundance within animal reservoirs is directly linked to assessing human health risk.

As obligate intracellular parasites, viruses evolve under continuous selective pressure. This pressure generates subpopulations with heritable traits that can give rise to new variants and, over time, new species. Across viral genera, structural plasticity in key proteins enables expansion of host range across mammalian, avian, and reptilian species. These structural adaptations also determine tissue tropism, receptor usage, and the resulting disease phenotypes, including respiratory, enteric, or systemic manifestations [11].

This evolutionary process occurs in humans and animal species, including wildlife and those central to agriculture and companionship. Completion of the viral life cycle frequently induces host pathology, initiating infection and imposing an immunological burden as the host mounts a defensive response. The physiological cost of infection can impair normal biological function while energy and resources are diverted toward recovery. When host defenses fail to control viral replication effectively, morbidity may progress to mortality, with potential escalation into broader public health consequences.

These interconnected dynamics are conceptualised within the One Health framework [6], which recognises the integrated health of humans, animals, and ecosystems. From this perspective, spillover events and emerging infections can be identified and mitigated before reaching pandemic scale. However, surveillance and diagnosis in animal populations present distinct challenges. For food production animals, intervention strategies are informed by economics and the relatively short animal lifespan. In wildlife populations, economics, accessing animals, and the inability to control migration patterns and movement are significant barriers to disease control. Viruses and other pathogens exhibit extensive biological diversity. They differ in genomic architecture, as classified by the Baltimore system [12], as well as in transmission routes, reservoir species, and pathological outcomes.

Coronaviruses provide a clear illustration of these principles [13]. Evolutionary history has established numerous animal species as coronavirus reservoirs. Domestic animals, wildlife, and humans commonly harbour low-pathogenicity coronaviruses and, more rarely, highly pathogenic strains. This broad host distribution reflects the capacity of coronaviruses for adaptive evolution in novel hosts, sustaining the ongoing risk of variant emergence and interspecies spillover. For example, human coronavirus

OC43 has a high homology with the endemic bovine coronavirus which affects cattle and is thought to have resulted from a spillover event about 100 years ago [14].

This evolutionary and ecological progression underpins the emergence of three epidemic or pandemic coronaviruses, SARS-CoV, MERS and SARS-CoV-2, within the past two decades [15].

1.1.3 The role of surveillance in preparedness

Robust surveillance systems can detect early signs of an emerging infectious disease and enable a rapid deployment of interventions, reducing the risk of wider spread. Surveillance tools include reporting of cases, epidemiology, genomic sequencing, use of sentinel sites, environmental monitoring, data-sharing and integration. Population-level surveillance provides intelligence on pathogen spread and evolution allowing assessment of disease severity and risk. Risk assessment and disease modelling informs public health interventions, such as vaccine deployment and wider use of diagnostics to inform behavior.

Global initiatives to support surveillance include the WHO's Mosaic Respiratory Surveillance Framework [16] and the Biosecurity Readiness through Intelligence, Data, and Global Engagement (BRIDGE) Alliance, with a drive to harmonise case definitions, laboratory protocols and share data.

1.1.4 Patient level diagnostics

In addition to surveillance benefits, diagnostics at the patient level inform the individual's clinical care and behaviour, reducing morbidity and the risk of onward transmission.

Early identification of infection allows patient isolation and infection prevention and control measures. To achieve this, diagnostics need to be widely and rapidly available,

and technologies such as lateral flow test, rapid molecular platforms, and multiplex assays are suitable [6].

With more severe disease, clinical care may be supported by knowledge of a patient's viral load and host response, for example informing use of therapeutics in a test-to-treat model. The continued effectiveness of therapeutics and vaccines as viral variants arise can be monitored by genomic analysis of patient samples aligned with clinical presentation and responses.

1.1.5 Challenges in pandemic preparedness for diagnostics

The critical value of diagnostics in pandemic preparedness is recognised, but significant challenges exist as highlighted in the 2025 IPPS-led Global Gap Assessment [7] produced with the Brown University Pandemic Center and FIND, the global diagnostics alliance.

The 100DM's ambitions for diagnostics are clear and include more rapid design, validation and regulatory approval for new assays enabling production at scale and global accessibility. This would enable rapid deployment of diagnostics at the early stages of pathogen emergence, allowing effective responses to reduce risk of spread. However, there remain weaknesses in technology, inequitable access and the health economic case [7]. Technology gaps highlighted include the requirement for disease-agnostic platforms, secure sample access and clarity of desired target product profiles. The current technical challenges are broad. Rapid development of a novel diagnostic for a new pathogen requires identification of antigenic or genomic targets, reagent design, analytical validation and regulatory approval. Rapid manufacturing requires scaled production of reagents, lateral flow strips, enzymes, conjugates, primers, plastics, instrumentation – all dependent on robust supply chains and dispersed geography.

Preparedness is significantly enhanced by using platform technologies that can be rapidly re-programmed. Multiplex platforms capable of detecting virus families would have the advantage of being more resilient to viral drift if diagnostics were designed around conserved genomic regions, such as the pancoronavirus RT-PCR assay for detection of all known coronaviruses reported by Vijgen et al [17]. Mutations causing changes in genomic sequences or structural proteins can render primer/probe-based or antigen-based detection ineffective.

For example, throughout the COVID-19 pandemic as variants arose the continued effective function of diagnostic assays was monitored (UKHSA). A late 2024 SARS-CoV-2 variant, BA.3.2 and related sub-lineages, have genomic deletions in ORF7A, ORF7B and ORF8, which causes PCR target failure in assays based on ORF8 [18]. This highlights the need for vigilance and acknowledgement that re-designing and re-validating diagnostic assays are slow and expensive processes.

The logistics of diagnostic test supply requiring cold chain storage, shelf-life constraints, transport, and customs processes all provide barriers to rapid and wide access. Diagnostic test technologies requiring a laboratory setting, skilled workforce, quality and data systems may also be challenging to expand rapidly. Alternatively, decentralised point-of-care tests which can be used more widely by non-specialists have a significant role to play for self-diagnosis.

During a pandemic, the focus on development and provision of diagnostics is supported by significant additional funding. However, as the crisis recedes, so does political attention and financial commitment, resulting in loss of diagnostic market stimulation, capacity and capability. The economic case for diagnostics is less easy to recognise than other elements of healthcare, so in times of constrained budgets

investment may become limited, leading to any urgent diagnostic response requirements being built reactively.

Organisations leading preparedness arrangements continue to promote improved technological approaches, such as multiplexed diagnostics and addressing gaps in the availability of rapid point-of-care tests. In addition, the broader issues of improved co-ordination, smarter financial models, harmonised regulatory pathways and sustained commitment are highlighted.

1.2 The importance of coronavirus disease

1.2.1 Important human coronaviruses and their pandemic potential

Coronaviruses are taxonomically classified within the family *Coronaviridae* and are divided into four genera, alpha-, beta-, delta- and gammacoronavirus [19]. These enveloped, single-stranded RNA viruses have large genomes (26 to 32kb) which are subject to both error-prone replication and high incidences of recombination leading to genetic diversification. Coronaviruses infect a wide range of species, human and animal, and through mutation and host receptor variation are prone to causing zoonoses [20].

Up to the end of the 20th century, human coronaviruses (HCoVs) were mainly associated with mild upper respiratory tract disease, particularly as a cause of 15% to 30% of common colds [21], although more vulnerable populations may suffer lower respiratory tract infections. Collectively, known as seasonal coronaviruses, HCoVs such as 229E, OC43, HKU-1 and NL63 circulate as endemic infections. Waning immune responses and viral mutation lead to frequent re-infections [22, 23].

However, in this century three significant zoonotic betacoronaviruses have emerged, demonstrating the pandemic potential of this virus family. Severe acute respiratory syndrome coronavirus (SARS-CoV) was first identified in China in 2002 and caused

over 8000 cases with a mortality rate of 10% [24]. SARS-CoV can cause severe illnesses including muscle pain, headache and fever, followed by cough, shortness of breath and pneumonia. Older adults over 60 suffered a higher mortality rate approaching 50%. The emergence of SARS-CoV was thought to be due to a spillover event between masked palm civets and humans, with a parent viral strain found in bats [25]. SARS-CoV infection spread across 29 countries over a few months and was brought under control by a combination of testing and patient isolation [26].

Middle East respiratory syndrome coronavirus (MERS-CoV) emerged in Saudi Arabia in 2012 associated with zoonotic infection derived from camels [15], again with a potential related strain found in bats. Most human cases have occurred in the Arabian Peninsula with subsequent outbreaks in Saudi Arabia in 2018 and South Korea in 2015 [27]. Human to human spread is rare outside healthcare settings, so MERS-CoV has low pandemic potential, however infection has a high mortality rate of ~35%. Symptoms include fever, cough, shortness of breath and pneumonia with gastrointestinal symptoms such as diarrhoea and nausea [28].

The most epidemiologically significant coronavirus is Severe Acute Respiratory Syndrome virus 2 (SARS-CoV-2) which emerged in Wuhan, China in late 2019 and caused the world-wide pandemic COVID-19 leading to over 7 million reported deaths [29]. SARS-CoV-2 is now considered endemic, and viral variants are continually emerging, challenging the control measures of diagnosis and vaccination [30] due to viral mutation and escape. SARS-CoV-2 is most closely genetically related to a bat coronavirus, although the exact zoonotic transmission route is unclear. Infections ranged from asymptomatic in 17% of cases to fatal in 10% of patients. Most people suffer mild or moderate symptoms such as coughs, sore throats, fatigue, headaches, but for those with other underlying conditions, COVID-19 can lead to more severe

respiratory disease, long term pulmonary, cardiological and neurological complications. The condition 'long COVID' is also recognised, characterised by long term fatigue. Human to human respiratory transmission results from virus loads reaching a peak in the pharynx approximately 4 days after infection [31-33].

These important demonstrations of zoonoses caused by coronaviruses and the genetic plasticity leading to host variations indicate that this family of viruses continues to pose a pandemic threat in future. Respiratory-borne infections have capacity for rapid onward transmission, leading to the potential for uncontrolled spread of infection and the resulting disease burden. With the more recent enhanced pathogenicity of SARS-CoV, MERS-CoV and SARS-CoV-2, there have been significant impacts on healthcare services and economic activity. Preparation for future risks posed by coronaviruses is important.

1.2.2 Animal diseases caused by coronaviruses.

Coronaviruses also have veterinary and agricultural importance, causing infection and disease in companion animals and those used in food production.

Two alphacoronaviruses infecting pigs, porcine epidemic diarrhoea virus (PEDV) and transmissible gastroenteritis virus (TGEV) cause severe enteric disease, particularly in piglets, leading to high mortality rates [34, 35]. A further alphacoronavirus causing disease in pigs is swine acute diarrhoea syndrome coronavirus (SADS-CoV), first reported in China in 2017 resulting from a spillover event from bats. A spike deletion of TGEV resulted in porcine respiratory coronavirus (PRCV) which causes a low pathogenic respiratory disease [36]. Transmission of enteric viruses is via the faeco-oral route, exacerbated by high density animal stocking systems.

Porcine hemagglutinating encephalomyelitis virus (PHEV) is a neurotropic betacoronavirus causing wasting disease or encephalomyelitis in piglets, resulting in

mortality rates of 20% to 50% [37]. Porcine deltacoronavirus (PDCoV) emerged in 2012 and causes enteric disease with a mortality of ~40% in piglets [35].

Bovine coronavirus (BCoV) is a betacoronavirus causing two types of disease, bovine respiratory disease complex and the enteric neonatal calf diarrhoea and winter dysentery in adult cattle. These diseases have varying mortality rates depending on co-infections and herd conditions. Transmission is faeco-oral or respiratory, again enhanced in intensive production systems [38, 39].

One of the most important avian coronaviruses is infectious bronchitis virus (IBV) which is in the gammacoronavirus genus. A significant poultry coronavirus, IBV shows high levels of genetic and antigenic diversity, particularly in the spike protein, which confounds immunity and vaccination [40]. IBV predominantly causes respiratory disease, resulting in coughing, sneezing and nasal discharge aiding transmission. Infection may also result in renal and reproductive tissues resulting in nephritis and reduced egg production. Mortality rates are variable depending on secondary bacterial infections [41].

In companion animals, the important viruses are feline coronavirus (FCoV) and canine coronavirus (CCoV), both alphacoronaviruses. There are two variants of FCoV, firstly feline enteric coronavirus (FECV) which causes mild enteric disease, and secondly, feline infectious peritonitis virus (FIPV) which is associated with immune-mediated disease affecting the vascular system, which is fatal if not treated with antivirals [42]. Canine coronavirus (CCoV) affects puppies causing enteritis, with low mortality in the absence of coinfections [43]. Transmission of these enteric infections are faeco-oral, again facilitated by close housing in kennels. Companion animals are also susceptible to SARS-CoV-2 illustrating the potential for bi-directional zoonotic transmission between humans and animals.

In addition to coronaviruses in companion animals and those of agricultural importance, of great significance is the role of wildlife as a major reservoir of diverse coronaviruses with potential for zoonotic transmission. Notably, bats harbour alpha- and beta-coronaviruses either asymptotically or with low levels of disease suggesting co-evolution over an extended time period [25]. Spillover events from bat infections, potentially through intermediate hosts, are associated with the emergence of SARS-CoV, MERS-CoV and SARS-CoV-2. Wild birds host delta- and gammacoronaviruses, with apparent low levels of morbidity and mortality, although migration may enhance transmission risk [44, 45]. The breadth of animal hosts affected by coronaviruses, coupled with zoonotic risk, highlights the importance of One Health surveillance and vigilance integrating human, animal and environmental risks.

1.2.3 Economic impact of coronavirus disease

The economic impacts of coronavirus disease are considerable. The SARS-CoV outbreak caused travel restrictions and loss of consumer confidence in addition to increased healthcare costs, resulting in overall costs of an estimated \$30 billion to \$50 billion [46]. SARS-CoV-2 caused world-wide impacts, increased healthcare costs, reduction in productivity due to lockdowns, widespread reduction in economic activity, and government-funded stimulus packages, all of which resulted in global GDP contracting by 3% to 4% [47]. In addition, COVID-19 led to educational disruption and mental health issues resulting in social and economic impacts. In the agricultural sector, pork supply has been affected by PEDV outbreaks in North America and Asia resulting in extensive levels of death in piglets [34]. Poultry and egg production are affected by IBV infection necessitating vaccination and biosecurity measures [48]. Outbreaks of disease also lead to movement and trade restrictions.

1.3 Coronavirus virology

1.3.1 Baltimore classification groups

Viruses are extremely diverse with a range of genome structures and strategies for replication. Many viruses must subvert translational systems within infected host cells and generate mRNA due to a lack of own ribosomes to synthesise proteins for viral progeny. The Baltimore classification system groups viruses into seven classes (I to VII) according to the nature of their genetic material and mechanism for generating mRNA.

1.3.2 Nidovirales order

The taxonomic *Nidovirales* order is used to group Class IV positive-sense single stranded RNA viruses causing respiratory, enteric, hepatic and vector-borne infections. This order classifies pathogens of major medical, veterinary and agricultural importance within several viral families including *Coronaviridae*, *Arteriviridae*, and *Tobamviridae*. These families classify viruses with a broad range of host species and transmission routes [25].

1.3.3 Coronaviridae family

The family *Coronaviridae* contains two sub-families, *Orthocoronavirinae* and *Letovirinae*. Whilst *Letovirinae* is a smaller sub-family, mainly used to classify viruses associated with amphibians and fish, it is sufficiently distinct from *Orthocoronavirinae* to justify sub-family separation. The *Orthocoronavirinae* sub-family is divided into four genera representing the more clinically important viruses infecting humans, other mammals and birds. Viruses from the four genera are termed alpha-, beta-, gamma- and deltacoronaviruses, which in turn contain sub-genera and numerous viral species (Table 1.1).

Genus (Orthocoronavirinae)	Subgenus	Lineage / Clade	Representative Species	Variants / Strains
Alphacoronavirus	Duvinacovirus	-	Human Coronavirus 229E (HCoV-229E)	Seasonal respiratory strains
	Setracovirus	-	Human Coronavirus NL63 (HCoV-NL63)	Seasonal respiratory strains
	Pedacovirus	-	Porcine epidemic diarrhoea virus (PEDV)	-
Betacoronavirus	Sarbecovirus	Clade 1a	SARS-CoV, WIV-1, Rs4231	-
	Sarbecovirus	Clade 1b	SARS-CoV-2 RaTG13	SARS-CoV-2 Variants of Concern (VOC): Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), Omicron (BA., XBB., JN.1)
	Sarbecovirus	Clade 2	RacCS203, RF1/2004	-
	Sarbecovirus	Clade 3	RhGB07, BtKY72	-
	Sarbecovirus	Clade 5	Rc0319	-
	Merbecovirus	-	Middle East respiratory syndrome Coronavirus (MERS-CoV)	Multiple regional clades (A, B, C)
	Embecovirus lineage	-	Human Coronavirus OC43 (HCoV-OC43)	Several genotypes (A–F)
	Embecovirus lineage	-	Human Coronavirus HKU1 (HCoV- HKU1)	Multiple genotypes (A, B, C)
Gammacoronavirus	Igacovirus	-	Avian infectious bronchitis virus (IBV)	Massachusetts,QX, Arkansas strains
Deltacoronavirus	Buldecovirus	-	Porcine delta Coronavirus (PDCoV)	-

Table 1.1: Taxonomy of *Coronaviridae* according to the International Committee on Taxonomy of Viruses (ICTV).

Coronaviruses are structurally spherical and contain the largest known RNA genomes (>26kb) within the taxonomic order *Nidovirales*. Genomic variants arising from mutation, recombination and insertion events leads to a broad range of viruses able to adapt to different hosts.

Contemporary taxonomic classification within the *Nidovirales* order is based on sequence-based taxonomy. The assignment of a virus into a separate species depends on the level of amino acid conservation within viral replicase domains encoded by ORF1a and ORF1b. A separate species is defined where these regions diverge by 10% or more. In addition, ongoing viral evolution can give rise to clades where a monophyletic group of viruses and their descendants arise from a single ancestor.

This breadth of coronavirus diversity provides a rigorous biological framework and model system in this study for evaluating diagnostic performance, enabling assessment of specificity and cross-reactivity across phylogenetically and antigenically distinct viral targets.

1.3.4 Sarbecoviruses

The sarbecoviruses are one of five betacoronavirus sub-genera differentiated by phylogenetic relationships in the replicase and spike genomic domains. The sub-genus contains important human pathogens such as SARS-CoV and SARS-CoV-2, alongside a broad group of diverse viruses associated with bats, particularly the horseshoe bat (*Rhinolophus spp*), which provide the main reservoir [49, 50]. SARS-CoV and SARS-CoV-2 both use the human host receptor ACE2, and whilst sarbecoviruses found in bats have a range of RBD structures, some are able to bind to human ACE2, highlighting zoonotic potential [51].

The predominant reservoir of sarbecoviruses is found in east and southeast Asia with the risks of spillover heightened by bat ecology, the trade in wildlife and contact between humans and animals. Coronaviruses are prone to mutation and recombination, thought to be the events giving rise to SARS-CoV and SARS-CoV-2 where sequence analysis shows relatedness to bat sarbecoviruses [50, 52]. Closely

related to SARS-CoV-2 are the BANAL viruses, which infect bat species and are able to use human ACE2 receptors, but which have not been found in humans. This demonstrates coronaviruses exist in bat reservoirs with the potential to infect humans [11]. As a further example, sequence analysis of UK bat samples discovered nine novel sarbecoviruses. Expression of viral spike proteins and screening of potential host receptors revealed one virus, RhGB07, which could sub-optimally use human ACE2, indicating potential for further mutation to enhance viral binding and entry and therefore zoonotic transmission [53].

Within the sarbecoviruses, there are two human epidemic-associated clades distinguished by genetic diversity in ORF1ab and other structural genes, in addition to different configurations of the RBD altering host range and transmissibility [54, 55]. The first is 1a, the SARS-CoV clade, which includes human and civet viruses, in addition to closely related bat viruses. The second is 1b, the SARS-CoV-2 clade which also includes closely related bat viruses, such as RaTG13 [50]. Clades 2 and 3 are predominantly sarbecoviruses associated with *Rhinolophus* bats across east and southeast Asia. A number of clade 3 sarbecoviruses have demonstrated hACE-2 receptor usage [56], however clade 2 viruses have large deletions within the receptor binding motif, which results in these viruses being unable to use ACE2 as their functional receptor [51, 56]. Clade 5 sarbecoviruses are associated with *Rhinolophus* bats found in Europe, which also do not bind to the human host cell receptor ACE2. These five clades represent the diversity of the sarbecovirus subgenus. Their propensity for mutation and recombination resulting in changes in host tropism illustrates their zoonotic potential and risk.

The sarbecoviruses therefore provide a good model for investigating the potential utility of novel diagnostic approaches.

1.3.5 Coronavirus genome organisation

Viruses classified within the *Orthocoronavirinae* family have a highly conserved genome organisation (**Figure 1.1**). The 5' end of the genome contains ORF1a and ORF1b which encode non-structural proteins associated with replication and proofreading. The 3' end of the genome encodes four structural proteins in the following order: spike, envelope, membrane, and nucleocapsid. There are also several accessory genes with immuno-modulatory roles. The large (26 to 32kb) positive-sense single stranded RNA genome is able to function directly as an mRNA encoding ORF1a and ORF1b (replicase genes) upon host cell entry [19].

Coronavirus SARS-CoV-2 genome structure showing non-structural, structural proteins and accessory proteins

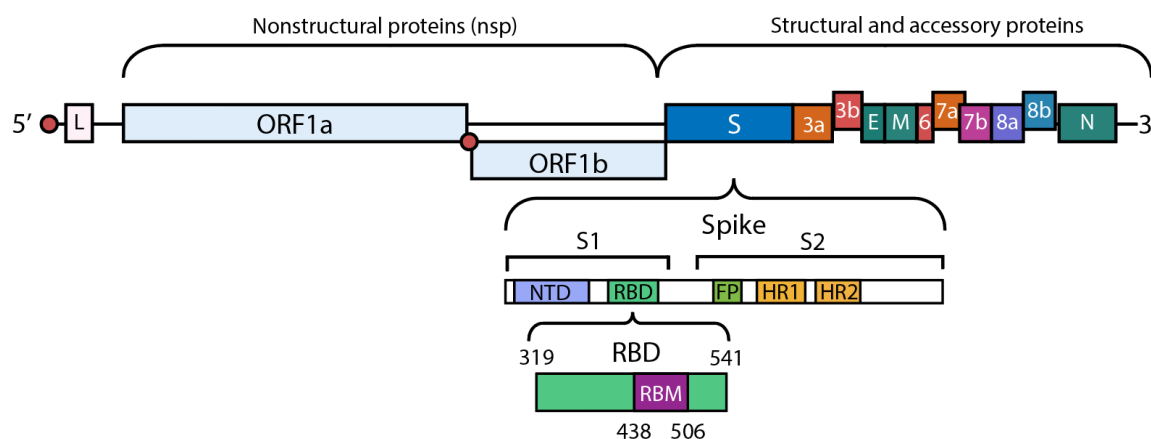


Figure 1.1: Coronavirus SARS-CoV-2 genome structure showing non-structural, structural proteins and accessory proteins. Adapted from [57].

1.3.6 Non-structural proteins

The two thirds of the genome at the 5' end containing ORF1a and ORF1b encode the replicase genes, which are translated into a polyprotein subsequently processed into 16 non-structural proteins which assemble into the replication-transcription complex (RTC). The RTC mediates RNA synthesis [58]. Enzymes involved in replication include the RNA-dependent RNA polymerase (NSP12), helicase (NSP13) and the proofreading 3'→5' exonuclease (NSP14), which is responsible for maintaining the

large RNA genome at a relatively low mutation rate of 10^{-6} to 10^{-7} substitutions per nucleotide per replication cycle [59].

Other non-structural genes encode accessory proteins which influence host immune responses, modulating pathogenesis, virulence, host adaptation and cross-species transmission [19].

1.3.7 Nucleocapsid protein

The coronavirus nucleocapsid (N) protein is 45 to 60 kDa and is an abundant structural protein which encapsidates viral RNA genome comprising two conserved folded domains, the N-terminal domain (NTD), primarily responsible for RNA binding, and the C-terminal domain (CTD), which allows the ribonucleoprotein complex to form [60]. Beyond its structural role in genome encapsidation, N protein is a regulator of viral replication and host–virus interactions. Although N is highly immunogenic, the host antibody response is not virus neutralising, due to the internal location of N within the virus particle and infected cell.

N is expressed early in infection and at significantly higher levels than most other viral structural proteins, increasing its detectability in clinical specimens such as nasopharyngeal swabs and saliva. This abundance makes N particularly well suited for antigen-based diagnostic platforms, including lateral flow immunoassays (LFAs), where analytical sensitivity is inherently limited by the absence of an amplification step. The combination of high intracellular expression, structural stability, and early production during infection enhances the diagnostic window for N-based detection. While conserved regions enable broad detection, sequence homology among endemic human coronaviruses can complicate assay specificity.

Despite these challenges, the N protein remains central to both antigen and molecular diagnostic strategies, balancing sensitivity with the need for resilience against viral evolution.

1.3.8 Membrane protein

As the most abundant virus structural protein, the coronavirus membrane (M) protein has a major role in virus assembly and structure. The M protein is 25 to 30 kDa and is a transmembrane glycoprotein which locates to the site of budding, the endoplasmic reticulum–Golgi intermediate compartment (ERGIC), interacting with N, envelope and spike proteins to enable virus assembly including packaging of RNA [19]. Virion curvature and morphology are also dependent on M protein. In addition, M protein has a role in virus-host interaction influencing innate immune responses [58].

1.3.9 Envelope protein

The least abundant coronavirus structural protein in mature virions is the envelope protein. It is a small transmembrane protein at 8 to 12 kDa, which is abundantly expressed in infected host cells, located at the ERGIC where it is involved in budding and virus assembly. The E protein also contributes to virus membrane curvature and membrane permeability [61].

In addition to the structural role, E protein also has an immunomodulatory role in the host and contributes to pathogenicity [61].

1.3.10 Spike protein

Coronavirus spike (S) proteins have a key role in the virus life cycle mediating host cell attachment and membrane fusion prior to entry [55]. As such they are the main contributor to host cell tropism and tissue specificity. The spike monomer, which is a type 1 transmembrane glycoprotein, assembles as a homotrimer to create functional spike proteins. The spike proteins protrude from the surface of virus particles giving

the appearance of a solar corona when viewed by electron microscopy, which lends the virus family its name. The spike protein is large at 180 to 200 kDa, containing between 1200 to 1400 amino acid residues and is heavily glycosylated and immunogenic, leading to neutralising antibodies therefore forming the basis of vaccines for COVID-19 [62].

The spike monomer consists of two subunits, S1 and S2, with S1 mediating host cell receptor binding and S2 which allows membrane fusion with the host cell. The S1 subunit contains the N terminal domain, which has a role in allowing conformational changes in the spike protein necessary for binding to the host cell receptor. Towards the C terminal domain (CTD) of S1 is the receptor binding domain (RBD), which contains the receptor binding motif (RBM) which interacts directly with the host cell receptor molecule. The S2 subunit contains the fusion peptide with the membrane-proximal region of S2 locating the protein into the virus envelope through the

transmembrane domain and cytoplasmic tail. An exemplar spike protein and the features of S1 and S2 in the coronavirus SARS-CoV-2 are shown in **Figure 1.2**.

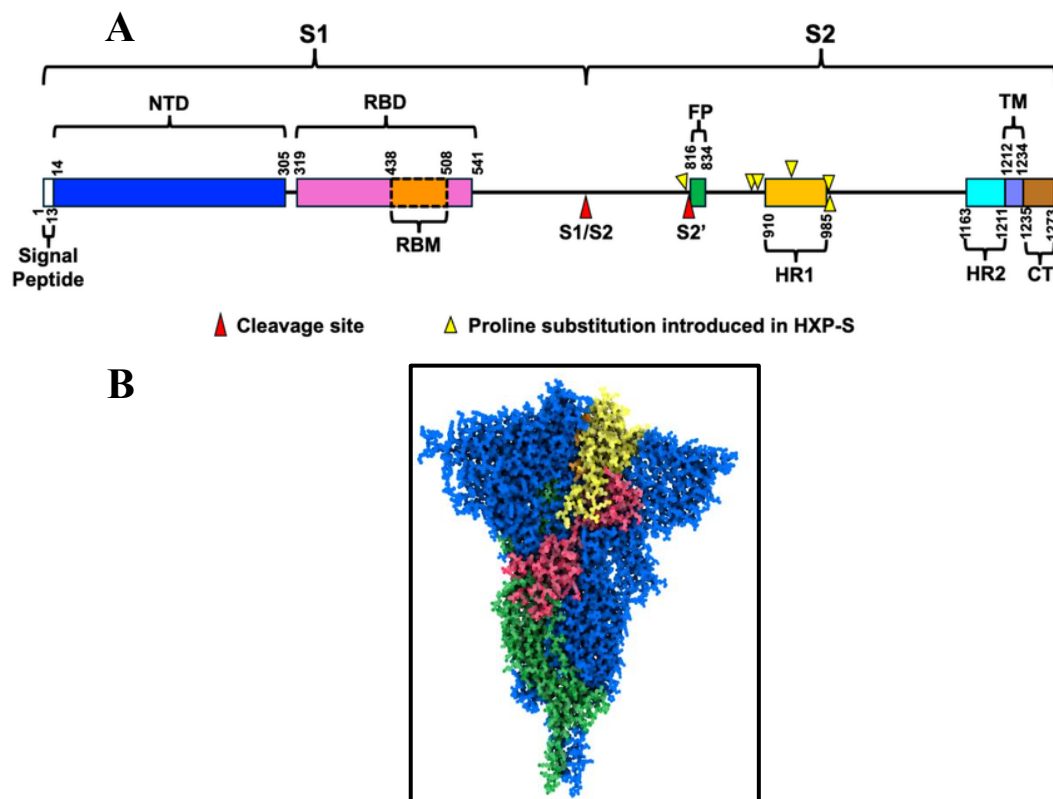


Figure 1.2: Domain organisation of the SARS-CoV-2 spike protein.

- A. Primary structure of the SARS-CoV-2 spike indicating the positions of key domains: the signal peptide (1–13 aa), the N-terminal domain (NTD; 14–305 aa), the receptor-binding domain (RBD; 319–541 aa), the receptor-binding motif (RBM; 438–508 aa), the fusion peptide (FP; 816–834 aa), the heptapeptide repeat sequence domains HR1 (910–985 aa) and HR2 (1163–1211 aa), the transmembrane domain (TM; 1212–1234 aa), and the cytoplasmic tail (CT; 1235–1273 aa). Adapted from Martinez-Guevara et al, 2026
- B. Representation of SARS-CoV-2 spike glycoprotein showing the location of the RBD (yellow – RBD, red-S1, green- S2, blue – second and third spike monomers)

As a trimer, S can exist in different conformations transitioning parts of its RBD between “down” (receptor-inaccessible) and “up” (receptor-accessible) configurations. In the down conformation, the CTD interacts with the RBD preventing binding to the host cell receptor. Alternatively, in the up conformation the RBM is exposed allowing host cell receptor binding.

There are a variety of host cell receptor molecules used by coronaviruses, with many strains infecting humans, such as SARS-CoV and SARS-CoV-2, using the angiotensin-converting enzyme 2 (ACE-2) molecule. Other host cell receptor molecules include DPP4 for MERS-CoV, aminopeptidase N for HCoV-229E, TGEV and FCoV, and sialic acid for IBV [63-66]. Coronavirus host-cell receptor interactions and variations through mutation are a key feature of zoonotic potential. The spike protein structure is broadly conserved throughout the coronavirus family as function must be retained to enable viral replication.

1.3.11 SARS-CoV-2 variants

Following the emergence in 2019 of SARS-CoV-2 and subsequent worldwide spread, numerous viral variants arose through mutation. These were sequenced, named and assembled into phylogenetic trees to assist with epidemiological tracking of the pandemic (**Table 1.2 and Figure 1.3**). Several naming systems were used, each with a different purpose. Firstly, the WHO classification of viral variants is based on epidemiological risk and phenotype rather than phylogenetic hierarchy, and assigns Greek letters (alpha, delta, omicron). Secondly, the Phylogenetic Assignment of Named Global Outbreak (PANGO) system classifies variants according to epidemiological impact and monophyletic clusters, thereby describing variant lineages associated with a specific outbreak [67]. The naming system uses letters and numbers to describe lineages, such as B.1.1.7, B.1.617.2, BA.1, BA.2.

The third system is Nextstrain, which is based on phylogeny and is used to track global-scale transmission of viral variants. This system uses a combination of the year of viral emergence and letters, such as 19A, 20I, 21A, 21K.

Nextstrain Clade	Pango Lineage	WHO Label	Emergence Date	Location of First Detection
19A	B	Wuhan-Hu-1-Hu-1 (reference)	December 2019	Hubei province, China
20I	B.1.1.7	Alpha (α)	October 2020	United Kingdom
21A	B.1.617.2	Delta (δ)	2021	India
21K	BA.1	Omicron ($\omicron$)	Late 2021	Botswana
21L	BA.2	Omicron ($\omicron$)	Early 2022	No single origin
23I	BA.2.86	Omicron ($\omicron$)	Mid 2023	Denmark and Israel
24A	JN.1	Omicron ($\omicron$)	Late 2023	United States
22F	XBB	Omicron ($\omicron$)	Mid 2022	No single origin
23F	EG.5.1	Omicron ($\omicron$)	Aug 8, 2023	China

Table 1.2: Progression of SARS-CoV-2 variants used in this study with their emergence date and location of first detection.

Cumulative confirmed cases - World (January 2020 - 2023)

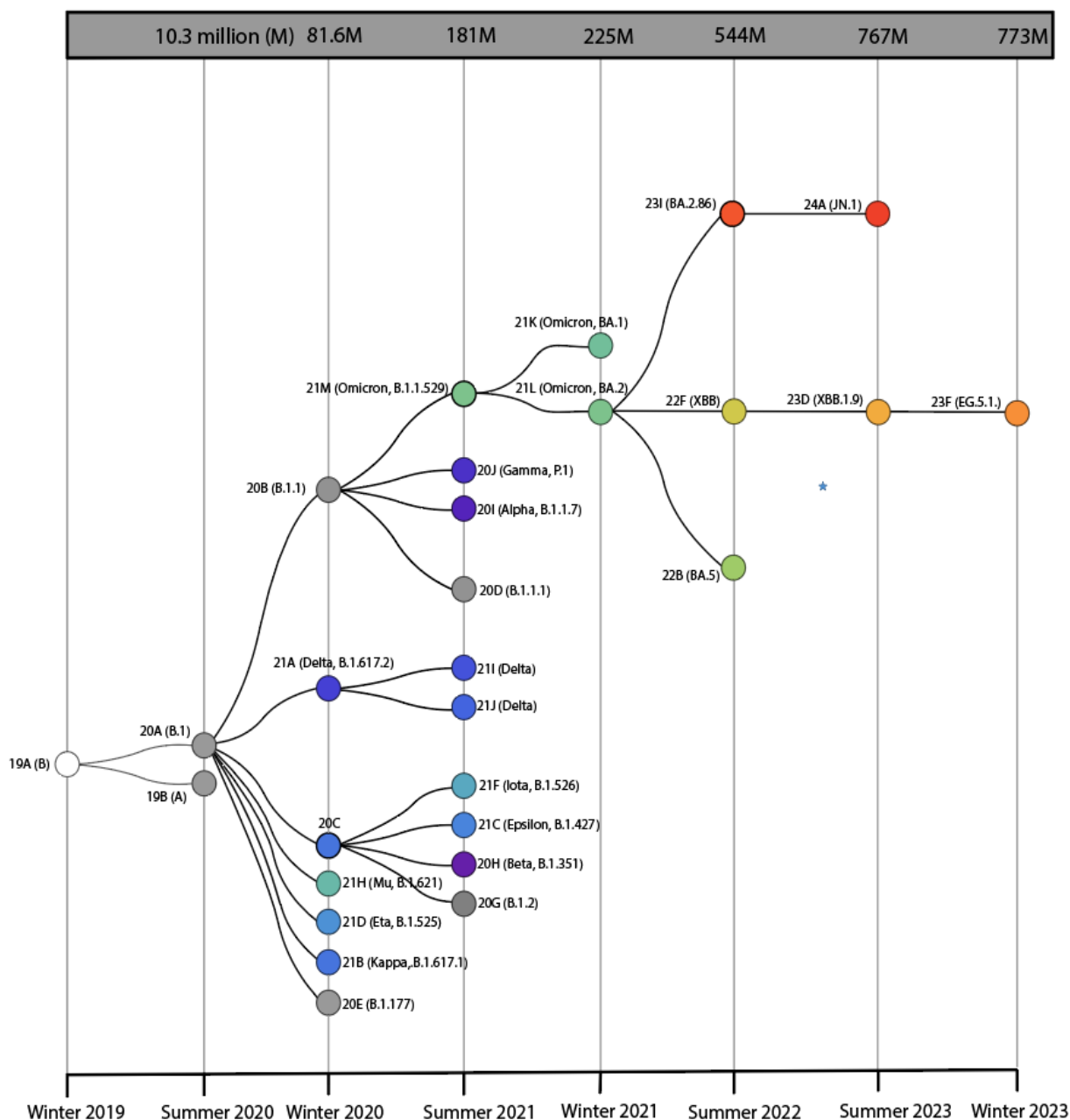


Figure 1.3: Phylogenetic tree of SARS-CoV-2 variant emergence and case numbers throughout 2020-2023. Illustration adapted from CoV variants demonstrating evolution of SARS-CoV-2 variants throughout the COVID-19 pandemic. Variants are labelled with their Pango and next strain variant names, with colours representing distinct variants.

As new variants arise, WHO considers changes in transmissibility, pathogenicity, and reduced effectiveness of diagnostics and vaccines and designates variants of interest (VOI) or variants of concern (VOC) according to potential impact. Particularly important is the continued efficacy of the intervention tools available to counter disease, including diagnostics, therapeutics and vaccines. As mutations arise and accumulate, viral variants may arise where viral proteins evolve away from the components on which the intervention tools were based, making them less effective. This study has investigated the potential of a particular type of molecular recognition elements with potential for diagnostic use developed to bind to SARS-CoV-2 Wuhan-Hu-1 S and RBD, to continue to recognise SARS-CoV-2 variants and wider members of the coronavirus family.

SARS-CoV-2 S protein mutations accumulate as variants emerge leading to alterations in amino acid sequence. The divergence between amino acid primary sequences of SARS-CoV-2 variants and wider coronaviruses' spike proteins can be expressed as a percentage and ranked by conservation (**Figure 1.4**).

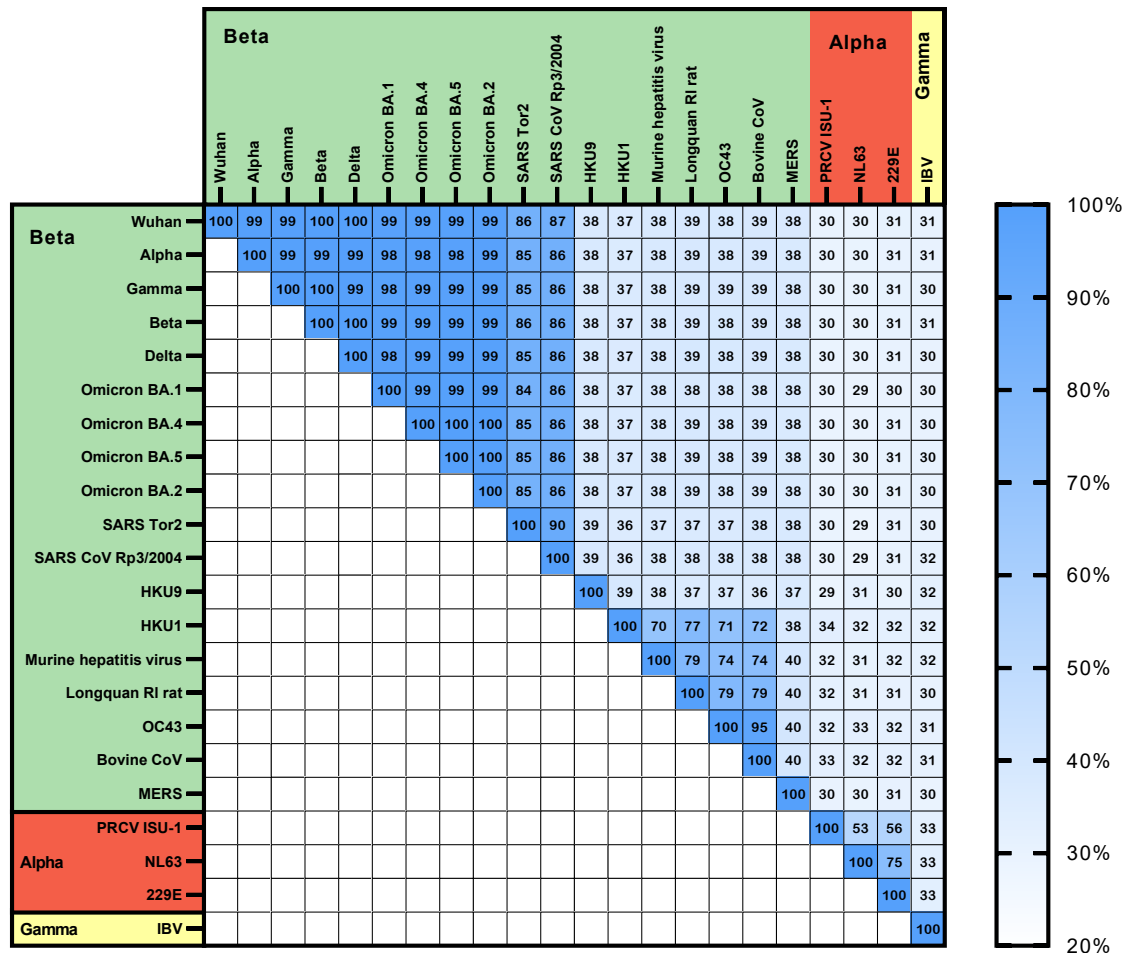


Figure 1.4: Divergent coronavirus spike proteins compared for amino acid homology with each other, expressed as a percentage and ranked by conservation.

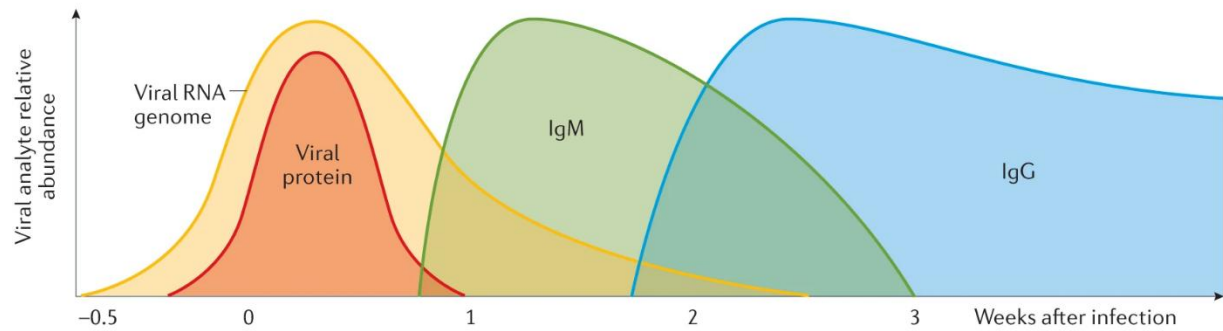
1.3.12 SARS-CoV-2 transmission

SARS-CoV-2 infection leads to high levels of virus production from epithelial cells in the upper respiratory tract. Transmission primarily occurs by viral excretion and expulsion via droplets and aerosols which are in turn inhaled by others or contaminate surface touchpoints. The number of onward infections caused by one infected individual is known as the basic reproduction number, R_0 , and varies according to SARS-CoV-2 variant, ranging from 2 to 12 [68]. A key feature of SARS-CoV-2

transmission includes high levels of virus shedding by both symptomatic and asymptomatic individuals [69].

The dynamics of virus production and excretion inform the required characteristics of an effective diagnostic (**Figure 1.5**). The timing post infection and the levels of excreted virus and viral components are key factors in the likely success of diagnosis and a resulting intervention, such as patient isolation, to reduce onward transmission. In addition, the site of virus excretion controls sampling methods which in turn inform whether diagnosis can be carried out by a member of the public or requires healthcare professionals, for example a nasal swab for viral respiratory infections can be self administered, but a blood draw cannot. Although the general profile of viral shedding throughout infection is established for SARS-CoV-2 (**Figure 1.5**), it should be noted that individual patients vary significantly [70].

a Viral analyte (RNA, protein and antibody) dynamics



b Infection stage and applicable test modalities

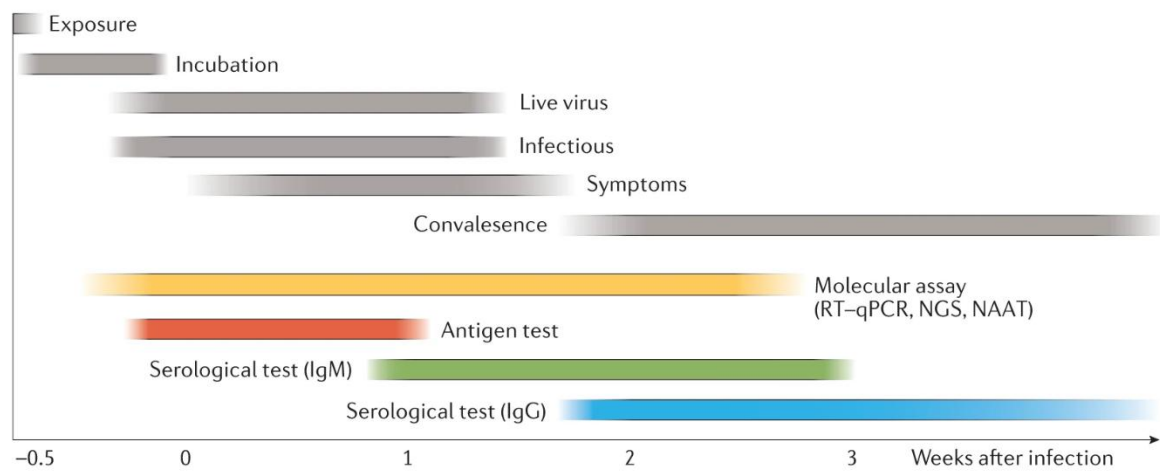


Figure 1.5: SARS-CoV-2 viral analyte production and applicable diagnostic test modalities post-infection.

Adapted from Mercer et al, 2021.

a | Shedding of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) RNA genome (yellow shading) typically increases rapidly following infection and peaks at the presentation of symptoms (although many patients can be asymptomatic) before gradually declining. Levels of viral proteins (red shading) also rapidly increase, albeit within a narrower window. In response to infection, the body produces IgM (green shading) and IgG (blue shading) antibodies, which persist for weeks to months.

b | The SARS-CoV-2 RNA genome can be detected by molecular assays, such as reverse transcription–quantitative polymerase chain reaction (RT–qPCR) before the development of symptoms. Serological tests can detect reactive IgG and IgM, which indicate past infection with SARS-CoV-2.

1.4 - Diagnostic technologies and application to SARS-CoV-2

1.4.1 Overview of Diagnostic Modalities

Diagnostic technologies for viral pathogens are defined by the type of biological target detected, the method of detection, and the real-world context in which they are used. The range of diagnostic approaches covers a spectrum of sensitivity, specificity, cost, turnaround time, scalability, and portability. The choice of diagnostic approach depends on the scientific or clinical requirement, such as mass screening, confirmatory diagnostics to inform interventions or surveillance to understand epidemiology and risk.

Biological targets include viral nucleic acid, viral proteins or host immune responses post infection. Diagnostic assays detecting viral nucleic acid, such as reverse transcription quantitative PCR (RT-qPCR) and loop-mediated isothermal amplification (RT-LAMP), involve an amplification step and are therefore more sensitive and able to detect low quantities of virus early in infection, for example, with SARS-CoV-2 [71]. These assays are used for confirmatory diagnostics and currently require a laboratory infrastructure and sample transport leading to turn-around-times (sampling to result) potentially of days.

Alternatively, assays based on viral antigen detection are less sensitive but may be more easily deployable at scale and can be used by members of the public. Antigen-based assays include ELISA and lateral flow devices (LFDs), which were used by millions of people during the COVID-19 pandemic to inform diagnosis and self isolation to limit infection transmission [72]. LFDs had the benefit of being cheap, scalable, portable and gave rapid results with important public health impact. Detection of host immune responses by techniques such as ELISA can be used to detect past infection and inform epidemiological studies, identification of host reservoirs and risk

assessment. During diagnostic assay development it is important that analytical performance is defined and validated to ensure suitability for purpose. Analytical performance includes assay sensitivity, specificity, precision and reproducibility.

Assay sensitivity is expressed as the limit of detection (LoD), the lowest concentration of virus that can be reproducibly detected. The LoDs of assays based on different viral target components are influenced by different variables. For example, nucleic acid assays are influenced by sample extraction efficiency, the presence of inhibitors and amplification kinetics. Diagnostics based on viral antigen detection are influenced by antibody affinity and signal detection thresholds [73]. Whilst lower LoDs may be beneficial for detecting low levels of infection, there is a potential trade off in the risk of non-specific reactions, particularly in amplification-based systems.

Diagnostic assay specificity is the ability to detect the intended target without detecting off-target viruses or other potential sample components. This is of particular importance where closely related viruses may contain partially homologous nucleic acid or proteins which bind primers, probes or antibodies giving potentially false positive results. Assay precision and reproducibility reflect the robustness of the assay carried out at different times by different operators, where consistent performance and repeatability is required. Once the analytical performance of a diagnostic assay is established, then the clinical performance also requires investigation to ensure the assay is robust in application in real world conditions. Variables encountered in the clinical context include sample complexity, the time post-infection and the prevalence of disease. The predictive value of the diagnostic assay is dependent on the prevalence of disease. The predictive value of a diagnostic test is calculated from the sensitivity and specificity of the assay and the application of these parameters to a population to be screened. The positive predictive value (PPV) is the probability that a

positive test result reflects a true infection and is not a false positive result. PPV increases as disease prevalence increases.

Conversely, the negative predictive value (NPV) is the probability that an individual with a negative test result truly does not have the infection. The NPV increases as disease prevalence decreases so negative results are more reliable when disease is rare. An example of this was RT PCR diagnosis of COVID-19 in patients admitted to hospital, which at a positivity rate of 10.2% gave a high NPV of 97.2% [74], however this NPV reduced to 96.8% as the positivity rate increased to 14%.

Whilst high PPVs and NPVs are desired they must be understood in the context of disease prevalence, to reduce risk of underdiagnosis.

The purpose of a screening assay is to initially detect a pathogen, or a closely related group, and the target may therefore be relevant to a broader group of related pathogens, for example based on conserved sequences. High analytical sensitivity is required to identify potentially positive samples [75]. Following screening, confirmatory assays may be used which are highly specific for the pathogen of interest and confirm true positive samples.

1.4.2 Nucleic acid amplification tests

Nucleic acid amplification tests (NAAT) are designed to increase small copy numbers of target genomes to millions of copies, amplifying the signal enabling diagnosis at the early stages of an infection where low viral titres are present.

The diagnostic gold standard for RNA viruses is reverse transcription quantitative polymerase chain reaction (RT-qPCR) as it is extremely sensitive and quantitative through cycle threshold (CT) values. Clinical diagnostics laboratories have established validated workflows, quality control procedures and a skilled workforce. Multiplex PCR, where multiple viral genes are targeted simultaneously, increases sensitivity and

specificity and also provides a level of diagnostic resilience in the presence of accumulating viral mutations. For SARS-CoV-2, various target genes are used for differing sensitivity and specificity. For example, the N gene is the most abundantly expressed viral transcript and therefore provides a high sensitivity target. Other viral genes have high specificity, such as ORF1ab containing RdRp and are used as confirmatory targets. This is in contrast to the E gene, which can be used in broad sarbecovirus detection as a screening target.

An alternative NAAT is reverse transcription loop-mediated isothermal amplification (RT-LAMP) which does not require a thermocycler and provides a colourimetric readout. These features potentially enable RT-LAMP to be used for diagnostic purposes in low-resource environments or in the home. However, there are some challenges, such as sample preparation, the requirement to maintain precise temperature control, and risk of contamination. RT-LAMP has comparable sensitivity levels as RT-qPCR and provides results rapidly.

Metagenomic next generation sequencing (mNGS) is a further NAAT approach which has a role in detecting novel or emerging pathogens from clinical samples and does not rely on pathogen-specific primers [76]. This unbiased approach relies on bioinformatics to compare sequences with reference genomic databases to identify pathogen-related signatures. Clinical mNGS is used in outbreak surveillance, epidemiology studies, and diagnosis particularly where the initial causative agent is unknown or rare. These included cases of Ebola [77, 78] and broader evaluation of pathogens in a clinical setting [79]. Deep sequencing allowed the tracing of genetic evolution of Ebola virus and the geographic spread of viral infection in the 2014 outbreak in West Africa [78]. The technology requires specialist equipment, a skilled workforce, and bioinformatics capability and is not currently used for high volume

routine diagnostics. Turnaround times are slower than RT-qPCR and sensitivity is lower.

These three NAAT technologies are complementary and used for different purposes: RT-qPCR for gold standard rapid, high volume routine diagnostics, RT-LAMP potentially for low resource settings, and mNGS for confirmatory typing, detection and discovery of novel or rare infections. The three approaches have a range of complexity, turnaround times and cost.

1.4.3 Antigen Detection Technologies

In contrast to NAATs where the viral target is nucleic acid, an alternative diagnostic approach is the detection of viral antigens present in patient samples. Antigen-based diagnostic assays are less sensitive than NAATs as there is no amplification step, so performance depends on antigen (viral) load which is generally highest during early symptomatic stages of infection [80]. To optimise sensitivity, assays use abundantly expressed viral antigens as target molecules, such as the N protein for SARS-CoV-2 detection. Detection depends on a tripartite sandwich format, where there is an antigen capture element, the viral antigen, and thirdly an antigen detection component linked to visualisation chemistry. The two most widely used antigen detection diagnostics are enzyme-linked immunosorbent assay (ELISA) and lateral flow tests or devices (LFT or LFD). ELISAs use immobilised capture antibodies and enzyme-conjugated detection antibodies to generate a colourimetric, chemiluminescent or fluorescent signal proportional to antigen concentration. This dual antibody recognition system provides a high level of specificity and detection is quantitative or semi-quantitative. ELISA procedures can be completed within hours with turnaround times dependent on sample collection and transport to diagnostic laboratories.

A Detection of viral proteins by lateral flow assays

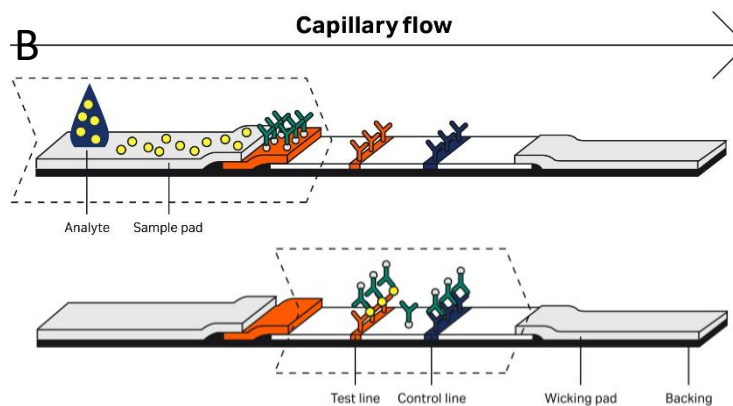
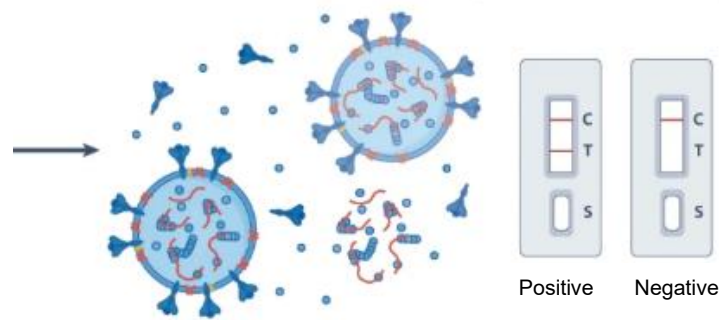


Figure 1.6: Illustration of the antibody-based lateral flow diagnostic test mechanism of action.

A. Lateral flow assays detect the presence of specific viral proteins in a patient sample. 'S' indicates where the sample is loaded, 'T' indicates the test line, and 'C' is the control line. Visible bands at both 'C' and 'T' indicate a positive test result, whilst a band at 'C' only is a negative result.

B. Initial stage. Viral antigen (yellow) starts to migrate in sample buffer by capillary flow, moving along the sample pad towards the gold nanoparticle-tagged detection antibodies (green). The test line is the location of the capture agent (orange) anchored to nitrocellulose. Anti-species antibodies (dark blue) are immobilised to nitrocellulose at the control line ready to capture unbound gold nanoparticle-tagged detection antibody.

C. End stage. As the sample and components migrate by capillary flow reaching the wicking pad, viral antigen is captured in a tripartite interaction (capture agent, viral antigen, gold nanoparticle-tagged detection agent) at the test line. Gold nanoparticles align in high density as a visible band indicating a positive test result. Unbound excess gold nanoparticle-tagged antibody continues to migrate and is captured by immobilised anti-species antibody at the control line. Gold nanoparticles align as a visible band at the control line, indicating that the test has performed as designed.

Scalable high-throughput assays are provided by automated ELISA platforms found in clinical diagnostic laboratories [81]. Lateral flow diagnostics use the same tripartite sandwich interaction principle as ELISA, where viral antigen is bound by a capture element and a non-competing detector element which is linked to visualisation chemistry. Monoclonal antibodies are widely used as capture and detector elements. However, in contrast to ELISA where the reactions occur within a plastic well, for LFTs the antigen in the sample binds labelled detection antibodies and migrates via capillary action across a nitrocellulose membrane to a capture line. As the sample migrates, gold nanoparticle conjugated detector antibodies are hydrated and released, resulting in binding to viral antigen if present. The antigen-antibody combination migrates along the nitrocellulose membrane, moving sequentially across specific antibody-coated capture bands that function as the test and control regions. A positive result is indicated via the formation of a dark test band and a dark control band within 10-30 minutes (**Figure 1.6**).

Development of LFTs requires optimisation of reagent distribution, concentration, and physicochemical conditions to ensure reliable antigen–antibody binding during assay operation. Critical reagents are spatially separated and must remain stable and desiccated throughout the product’s shelf life. Upon contact with a liquid sample, these reagents must rapidly rehydrate, solubilise, and regain full biochemical activity.

The WHO sets target product profiles (TPP) for lateral flow diagnostic tests, including desired performance in specificity of greater than 99% and sensitivity greater than 80% [82]. This standard is the threshold that should be applied to new diagnostic applications to ensure tests are useful to end users. Diagnostic test characteristics of SARS-CoV-2 LFTs were shown to have a lower limit of detection (LLOD) equivalent to

10,000 RNA copies/mL equivalent to a Ct of 25-28 in RT-qPCR using SARS-CoV-2 specific primers [83].

Although LFTs are less sensitive than RT-qPCR, they do not require any specialist equipment, are relatively cheap, and can be deployed at scale as was the case for mass home testing during the SARS-CoV-2 pandemic. LFTs can detect viral loads associated with a high risk of onward transmission of infection, which is a critical indicator to enable interventions such as self-isolation to reduce population-level risk (**Figure 1.5**).

A key consideration for test sensitivity is the quantity of antigen recovered during sampling. A study showed variability of results between research scientists, clinicians, and untrained users in rapid antigen tests, as well as at various stages of pathology and viral shedding. For reference, the quantity of total recovered antigen via swabbing has been linked to infectivity, with evaluation studies of antigen lateral flow tests focusing on sensitivity performance at 10,000 (10^4) (Ct > 30) and 100,000 (10^5) (Ct $\approx$ 25–30), viral copies/mL [84].

Lateral flow tests used in their millions during the COVID-19 pandemic were based on the detection of nucleocapsid protein in patient samples [85]. Nucleocapsid protein is an abundant SARS-CoV-2 viral protein providing high levels of assay sensitivity [86] and is produced early in infection in the upper respiratory tract facilitating early diagnosis and therefore interventions to reduce transmission risk. There is good alignment between rapid antigen detection tests, such as LFTs, and RT-PCR positive results where Ct values are below 25, but less so at higher Ct values where there are lower RNA viral loads. Due to the greater sensitivity of RT PCR, infection can be detected 1 to 2 days earlier than by LFT, and the peak of LFT positive results occurs within 7 days post onset of symptoms (dpos). After the acute phase of infection at

approximately 10 dpos, LFT positive results decline as does the presence of recoverable infectious virus.

Nucleocapsid is strongly genetically conserved, particularly at the N- and C-terminal domains, with >90% homology at the amino acid level [87], [88] between SARS-CoV-2 variants allowing continued recognition by capture and detection antibodies therefore providing a resilient assay. A small number of LFT tests are commercially available based on SARS-CoV-2 spike protein [85], although spike protein is less abundant and has a higher mutation rate than nucleocapsid due to immune pressure [89]. In summary, ELISA provides a higher analytical performance requiring a laboratory, and LFTs provide a rapid, mass deployable diagnostic.

1.4.4 Antibody detection with serological diagnostics

The detection of host generated antibodies following infection is useful for surveillance, clinical diagnosis and monitoring vaccine efficacy. Immunoglobulin classes such as IgM, IgG and IgA can be measured and used to indicate the stage of infection. IgM is associated with early infection, whereas IgG levels rise several days post-infection. Secretory IgA levels also rise early post-infection produced at mucosal surfaces associated with initial infection, neutralising virus to reduce infection and transmission. IgG production indicates humoral response maturation with the role of longer-term systemic immunity [90].

ELISA is the most commonly used high-throughput technique for quantifying post-infection antibody levels and uses an abundantly expressed viral antigen to capture antibody in patient samples. Lateral flow assays can also be designed to measure antibody levels, with the benefits of rapid, point-of-care use, but with lower sensitivity than ELISA.

1.4.5 Emerging diagnostic technologies

The COVID-19 pandemic stimulated the development of alternative diagnostic platforms with the aim of scalability, high sensitivity, short turnaround times and decentralised application. Three developing technologies are described below.

CRISPR-based technology is based on directable nuclease activity, which can be targeted to specific viral RNA sequences for diagnostic applications. Target recognition enables cleavage of reporter molecules with fluorescent or lateral flow readouts. The technology is sensitive and able to distinguish viral variants and does not require thermocycler equipment [91-93].

Digital PCR partitions samples into high numbers of microreactions enabling viral RNA quantification without standard curves. This approach enables improved sensitivity at low viral RNA copy numbers compared with RT-qPCR, which is useful for early diagnostics [94]. The technology requires a laboratory setting.

Microfluidic platforms (lab-on-a-chip devices) can be used to miniaturise reactions, integrating sample preparation, amplification and detection, especially with isothermal or CRISPR approaches. This diagnostic approach can be encapsulated in portable automated cartridges suitable for decentralised applications [95].

1.4.6 Use cases of LFDs and design

The core use case for lateral flow devices covers three areas, (a) the need for decentralised testing, (b) in resource limited environments and (c) to support an urgent need to expand testing capacity beyond the limits of a normal centralized diagnostic service.

Pre-COVID, the most prominent use of lateral flow tests was in human pregnancy testing, where combinations of antibodies detect the hormone glycoprotein human chorionic gonadotropin (hCG) in urine. This is an example of testing capacity being

available on a commercial basis, where individuals can determine their status independently of healthcare providers, offering accessibility and well as privacy of pregnancy status. LFDs satisfied an urgent use case in the COVID-19 pandemic. Following validation, monoplex LFD tests against SARS-CoV-2 could inform individuals of their infection status allowing informed decisions about self-isolation. Widespread home-based use of LFDs was introduced in response to regional diagnostic laboratories being overwhelmed with the growing case numbers, providing rapid testing that could be performed without training. In addition to monoplex LFTs which detect a single antigen, multiplex assays could be developed to detect a range of pathogens with a common clinical presentation. For example, a panel encompassing antigen detection for influenza, SARS-CoV-2, seasonal coronaviruses and Respiratory Syncytial Virus would inform clinical responses to respiratory infections.

1.5 Viral protein molecular recognition elements

Diagnostic assays detecting viral protein components rely on the availability of molecular recognition elements (MREs), or affinity ligands, able to bind viral proteins with high specificity. This enables viral antigen capture and detection. MREs include antibodies and their derivatives, engineered protein scaffolds, and aptamers, all of which bind to viral antigen epitopes.

1.5.1 Antibodies

The most commonly used MREs are monoclonal antibodies used in a diverse range of laboratory assays to identify protein targets, both within complex biological samples (such as flow cytometry or ELISA) and within cell lysate samples (Western blot, immunoprecipitation), as well as in epitope mapping by blocking target regions.

Binding of antibody to antigen is mediated via the paratope and epitope sites respectively, and epitopes may be linear or conformational.

Antibody molecules are produced by B cells following viral infection and undergo class-switch recombination and affinity maturation in germinal centres. B cells can be screened for reactivity to antigens, isolated and hybridised with carcinoma cells in vitro to produce immortalised populations of identical cells capable of producing single clonal (or monoclonal) populations of antibodies. The generation time for monoclonal antibodies requires immunisation of animals to develop relevant B cells, followed by several weeks for cell hybridisation, cloning and banking. Therefore, an estimated timeline of 4-6 months for generation of purified antibodies is common. In addition to the time required to develop a reliable, quality assured supply of monoclonal antibodies, they are large proteins and challenges remain in long-term storage, batch variability, high costs, and the risk of heat denaturation.

In addition, there is a risk that binding specificity may be lost in the event of a rapidly mutating viral target, although some monoclonal antibodies bind to conserved viral sequences conferring broad binding application to divergent strains. Examples include the mAbs VIR-7229 [96] and WRAIR-2063 [30], which show cross-reactivity against RBDs across clade 1a (SARS-CoV viruses), clade 1b (SARS-CoV-2), clade 2 (non-ACE2-utilising bat coronaviruses), and clade 3 (African and European sarbecoviruses), and which exhibit binding to class 5 epitopes within the RBD as cryptic conserved epitopes.

Some of the disadvantages of full-length antibody molecules can be overcome by employing antibody-derived molecules, such as Fab fragments, single chain variable fragments (scFv) or nanobodies for diagnostic or therapeutic purposes.

Fab fragments are generated by proteolytic cleavage of full-length immunoglobulin molecules, retaining the antigen binding site but removing the Fc region and reducing non-specific binding in diagnostic assays [97]. Recombinant antibody fragments, such as scFv, retain antigen binding capacity and can be fused to reporter molecules. Microbial recombinant production systems are frequently used following antibody variable region selection through phage display for example [98]. Nanobodies are single heavy chain only antibodies found in camelid species. These antibodies are small, and highly stable at higher temperatures.

1.5.2 Engineered Protein Scaffolds

A separate class of MREs are engineered protein scaffolds which are small affinity molecules designed to bind to target proteins. These small (6 to 20 kDa) unglycosylated scaffold molecules can be manufactured in microbial recombinant production systems. Particular target binding specificity is determined by generating diverse surface residues within the structural framework, followed by selection through display technologies, such as phage. Structural target protein characterisation and computational engineering provide a rational basis for MRE design and targeting conserved epitopes for diagnostic resilience.

There are a range of scaffold classes such as designed ankyrin repeat proteins (DARPs) [99], affibodies [100], monobodies [101], anticalins [102] and affimers. Advantages of these small scaffolds include high thermal stability, and dense surface immobilisation for diagnostic assays.

1.6 Aptamers as diagnostic tools

1.6.1 Aptamers

Aptamer technology emerged as protein-binding ligands based on incremental enrichment of RNA structures with preferential binding to small organic dyes and T4

DNA polymerase protein respectively, as described in two seminal papers in 1990 by Ellington & Szostak [103] and Tuerk & Gold [104]. Based on this discovery, their application expanded to ssDNA, and the development coined the phrase used for aptamer generation called SELEX (systematic evolution of ligands by exponential enrichment) (**Figure 1.7**).

Aptamers can be made from single stranded DNA or RNA, with nucleotide composition differing slightly. DNA aptamers consist of nucleotides A (adenine), G (guanine), and C (cytosine) and deoxythymidine (dT), with deoxythymidine replaced by uracil in RNA aptamers. Nucleic acid aptamer structures are generated via SELEX from random sequence libraries over rounds of binding and PCR amplification, with selection to target protein structures and counterselection to closely related non-target structures (**Figure 1.7**). Due to this rigorous selection process, aptamers have been reported to perform similarly in binding affinity and specificity to monoclonal antibodies [105]. Aptamers are small molecules, 20-100 nucleotides in length and 3-20 kDa in weight, and bind to target analytes as a result of their unique three-dimensional shape via binding through Van der Waals forces, hydrogen bonding, stacking of aromatic rings, salt bridges, other electrostatic interactions, and shape complementarity. Research on ssDNA aptamer binding also indicates that these small molecules engage with physically small epitopes that may be sterically inaccessible to larger antibodies, highlighting differences in size between aptamers and monoclonal antibodies (~20 kDa vs. ~150 kDa IgG).

Significant benefits of aptamers for diagnostic applications in comparison to monoclonal antibodies are their speed of generation, batch-to-batch consistency, orders of magnitude reduced cost and stability. The speed of generation is a critical factor in rapid response and with advancements in automated production of aptamers,

integrated robotic systems can now generate selected candidates within 48 hours, starting from the stage of purified antigen/immunogen produced recombinantly from published genome sequences [106]. At the start of the SARS-CoV-2 pandemic, the complete viral nucleotide sequences were published within weeks of first discovery, enabling a global community of scientists to begin characterising the virus. Viral structural proteins were rapidly cloned and expressed, providing materials required for the SELEX process. In addition, due to available sequence information at each stage of SELEX, parallel in silico binding experiments can be performed with DNA sequences and structural proteins aiding in the rapid selection of epitope specific aptamers [107].

In addition, the production of aptamers, in contrast to monoclonal antibodies, avoids the use of animals to generate an immune response, saving time, meeting the 3Rs agenda (reduce, refine, replace) and facilitating consistency in production between batches. Aptamers as small nucleic acid molecules can possess chemical modifications, making modified aptamers more heat resistant and stable to pH stress than monoclonal antibodies which may be an advantage to field applications. With these advantages, aptamers have the potential to replace monoclonal antibodies as MREs in the generation of rapid response diagnostics. Lateral flow diagnostics utilising aptamers have been demonstrated across a broad range of disease and environmental analytes within medicine, agriculture, food and environmental safety. Proof of concept has been shown for the use of aptamers in LFDs for several infectious agents, including influenza virus, *Salmonella typhimurium*, *Listeria monocytogenes* and *E. coli* [108]. In addition to LFDs based on aptamer pairs, the potential for hybrid recognition elements of a Mab and an aptamer combination in influenza detection has been reported by Thao et al 2017. [109]. However, this approach potentially negates

the benefits of aptamers as described below. Aptamer-based LFDs offer the potential for multiplexed, multi-pathogen, affordable, easy-to-use field-deployable diagnostics for detection of emerging infection threats in animal reservoirs or diagnosis of human infections.

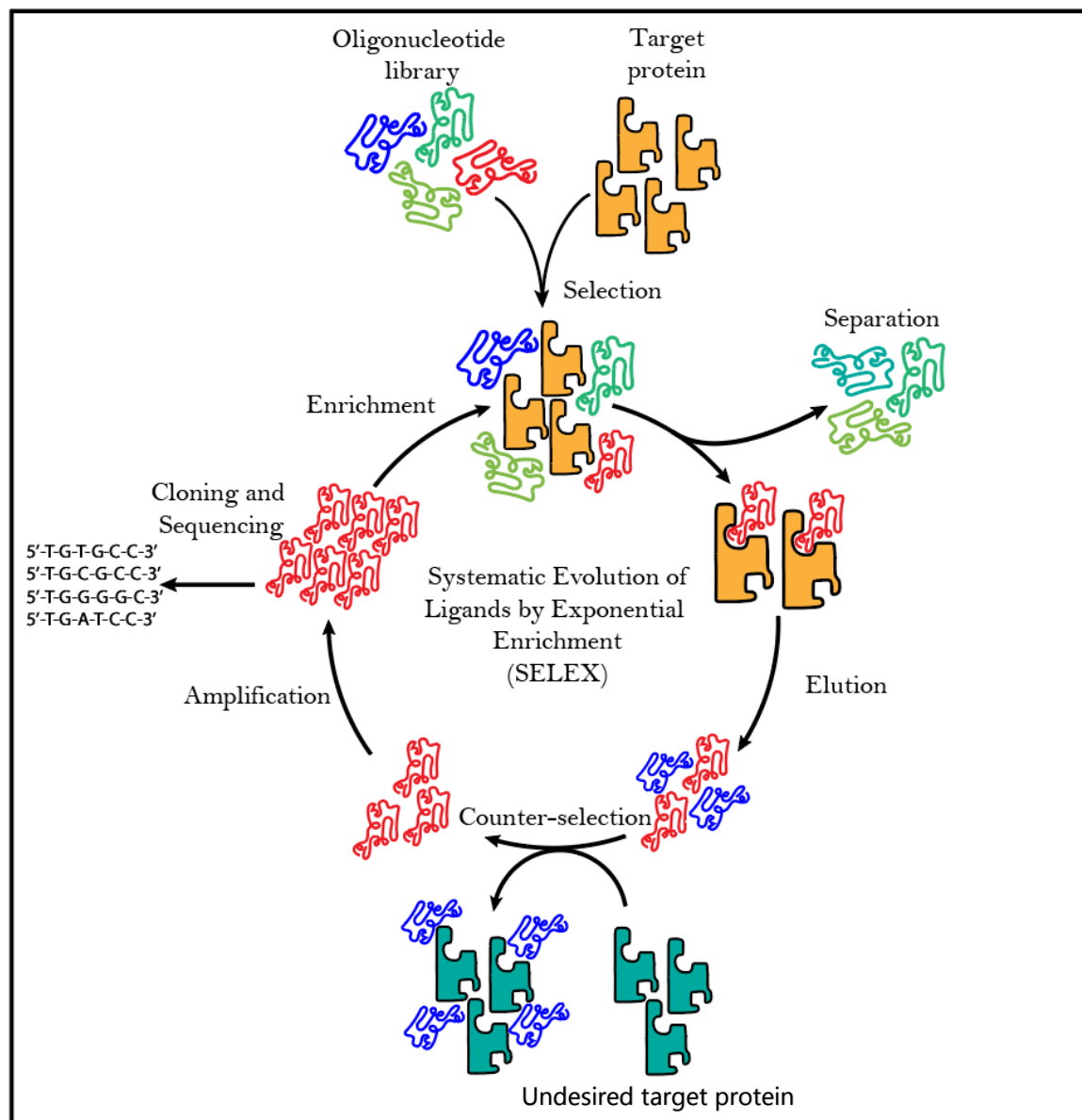


Figure 1.7: SELEX process adapted from Bognar et al, and Zhou et al, 2017 [110]

An oligonucleotide library is created of random sequences which are then allowed to bind to the target antigen. Unbound oligonucleotides are removed from the process. Bound oligonucleotides are eluted from the target antigen and allowed to bind to an unrelated undesired protein in a round of counter-selection. In this case, the bound un-wanted nucleotides are removed from the process. The remaining oligonucleotides are amplified, cloned and sequenced. Further selection rounds can be undertaken to obtain an enriched population of oligonucleotides with improved specificity for the target protein.

1.6.2 SOMAmers

Slow off-rate modified aptamers (SOMAmers) are a form of aptamer generated from an extensive pool of short single stranded DNA sequences comprised of nucleotides A (adenine), G (guanine), and C (cytosine) in addition to an unnatural base deoxythymidine (dT). This thymidine nucleoside possesses protein like functional groups bound to a uniform 5' position, providing enhanced properties in ligand efficiency, metabolic stability and epitope coverage compared to aptamers [111].

In addition to the SELEX process based on affinity to target antigens, SOMAmers are also exposed to a kinetic challenge step whereby protein/DNA complexes are incubated in the presence of the polyanionic competitor dextran sulphate, selecting for SOMAmers with slow off-rate from targets forming molecules with high affinity binding. An established application of ssDNA ligands within the founding company SOMAlogic is a proteomics platform, SOMAscan, which enables these structures to simultaneously detect up to 7,000 disease biomarkers from a patient serum sample. Additionally, SOMAmers are effective as therapeutic molecules, achieving regulatory approvals for macular degeneration in the form of Pegaptanib [112]. Equally, they have been identified for their capacity to neutralise in vitro SARS-CoV-2 variants of concern via binding to RBD of the spike glycoprotein [111].

1.6.3 SELEX Limitations

The SELEX process for aptamer and SOMAmer generation is subject to several limitations. Theoretically, the most significant risk is applying too many consecutive negative-selection rounds against related antigens. This causes loss of aptamers with biologically useful cross-reactivity. In a pan-coronavirus context, aggressive counter-selection against related coronaviruses risks removing aptamers directed at conserved RBD subdomains, rather than only removing strain-specific, undesired

binders — which directly undermines the pan-reactive design goal. This can be countered by benchmarking binding performance before and after each negative-selection round and rolling back to the prior pool if diversity or conserved-epitope binders collapse.

This compounds with a second issue. PCR amplification bias caused by GC content and secondary structure which leads to some sequences to out-amplify true high-affinity binders, enriching for "PCR winners" over aptamers with the best target affinity. This is mitigated by minimising cycle number and monitoring amplicon distribution by qPCR between rounds.

1.7 Aims of the study.

Between 2020 and 2024, the majority of commercially available rapid SARS-CoV-2 LFT diagnostics were based on monoclonal antibody technology. However, in the context of the 100-day mission, alternative binding molecules may offer advantages over conventional monoclonal antibody-based platforms. This research focused on the early development of a lateral flow device utilising the MREs ssDNA SOMAmers as a rapid, scalable, portable mass-testing platform for the detection of viral antigens as a screening or confirmatory diagnostic.

The hypothesis of this study was that aptamer molecules (SOMAmers) can replace monoclonal antibodies as capture and detection molecules in a diagnostic test for coronaviruses of human and veterinary importance.

Research was undertaken to evaluate a library of SOMAmers provided by the industrial partner (SomaLogic) that had been raised against the spike and nucleocapsid structural proteins of SARS-CoV-2 Wuhan-Hu-1 strain.

Research objectives are as follows:

Objective 1 – To evaluate the ability of the SOMAmer library to detect a broad range of coronaviruses as a potential screening assay or a variant specific diagnostic. To identify binding between the SOMAmer library and viral RBD or spike glycoproteins derived from:

- a) Sarbecovirus subgenus representatives (SARS-CoV-2 variants, Clade 1a,1b, 2, 3 and 5)
- b) Non-SARS-CoV-2 betacoronavirus H-CoVs.
- c) Representative human and animal alpha and gammacoronaviruses.

Objective 2 – Determine competition between SOMAmers binding to epitopes present within viral antigens. Identify non-competing pairs of SOMAmers that can function in the detection and capture roles for application in lateral flow diagnostic prototypes.

- a) Establish self-self SOMAmer binding inhibition against a range of SARS-CoV-2 and sarbecovirus RBDs.
- b) Establish competitive binding inhibition or non-competitive permissive binding between SOMAmers, each in both immobilised and in blocking roles against a range of SARS-CoV-2 and sarbecovirus RBDs.
- c) Establish kinetic performance of SOMAmer candidates binding to target antigen.

Objective 3 – Establish an early-stage lateral flow test prototype using non-competing SOMAmer pairs.

- a) Establish gold nanoparticle (Au-NP) tag detection SOMAmers are able to bind to target antigen and act as visual signal reporters.
- b) Demonstrate tagged SOMAmers function to retain binding to target antigen on nitrocellulose surface.

- c) Combine detection and capture SOMAmers together to show functional tripartite interaction to SELEX RBD antigen target.
- d) Demonstrate binding specificity using non-specific binding control SOMAmers using SELEX RBD antigen target.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 SOMAmer Libraries

2.1.1.1 Main SOMAmer library

The main SOMAmer library was provided by SomaLogic (**Table 2.1**). Full details are provided in **Appendix 1**.

Number of SOMAmer pairs	SELEX target
2	SARS-CoV nucleocapsid
1	SARS-CoV-2 nucleocapsid
9	SARS-CoV-2 receptor binding domain
5	SARS-CoV-2 S1
31	SARS-CoV-2 S1/S2 ectodomain

Table 2.1: Main SOMAmer library.

Each SOMAmer was provided as a pair, either a biotin-tagged or amine version. Each stock was supplied at 250 μ M.

Selected SOMAmers with thiol or SENPAH 5' proximal end groups (supplied by SomaLogic) were used for LFT prototype development.

2.1.1.2 SOMAmer size analysis by gel electrophoresis

SOMAmer samples were diluted to 316 nM in formamide/TBE buffer and 6x loading dye (BIORAD #1660401EDU), heated to 95°C for 3mins, then placed on ice. Samples were resolved on a gel made using the following reagents 40% acrylamide, 2% bisacrylamide, 10% TBE, 10% APS, TEMED, 7 M urea. Molecular weight markers were provided by a ssDNA oligonucleotide ladder (IDT #51-05-15-01), combined with formamide/TBE and loading dye before loading. Electrophoresis was run for 1hour 15 minutes at 100V in TBE buffer for ssDNA migration. Bands were visualised with Sybr Gold (Thermofisher #S11494) diluted 1/10000 from stock with TBE buffer, and an Odyssey M imaging system at 495 nM emission.

2.1.1.3 Second SOMAmer library

During the study, a further small library supplied by SomaLogic, referred to here as the second SOMAmer library, was made available which had previously been evaluated as potential therapeutic agents against SARS-CoV-2 variants (**Table 2.2**). These further SOMAmers extended the materials available for study.

Nomenclature	Label	SELEX target
SL1107_18	A1	SARS-CoV-2 receptor binding domain
SL1108_18	A2	SARS-CoV-2 receptor binding domain
SL1111_20	A3	SARS-CoV-2 receptor binding domain
SL1114_18	A4	SARS-CoV-2 S2
SL1115_18	A5	SARS-CoV-2 S2

Table 2.2: Second SOMAmer library

Each SOMAmer was biotin-tagged and supplied at 250 μ M [111]

2.1.2 Recombinant viral proteins

Genus (<i>Orthocoronavirinae</i>)	Subgenus	Lineage / Clade	Representative Species	Variant	Structural Protein	Source
Alphacoronavirus	Duvinacovirus	-	Human Coronavirus 229E (HCoV-229E)	-	Full Length Spike (FL-S)	Sinobio (40605-V08B) (Purchased)
Alphacoronavirus	Setracovirus	-	Human Coronavirus NL63 (HCoV-NL63)	-	FL-S	Sinobio (40604-V08B) (Purchased)
Alphacoronavirus	Pedacovirus	-	Porcine respiratory coronavirus (PRCV)	ISU1	FL-S)	Pirbright Institute – Mucosal Immunology group (Gifted)
Betacoronavirus	Sarbecovirus	Clade 1a	SARS-CoV	TOR2	FL-S	Sinobio (40634-V08B) (Purchased)
Betacoronavirus	Sarbecovirus	Clade 1a	SARS-CoV,	-	Receptor Binding Domain (RBD)	Dr Jack Tan – University of Oxford (Gifted)
Betacoronavirus	Sarbecovirus	Clade 1a	WIV-1, Rs4231, SHC014	-	Receptor Binding Domain (RBD)	Viral Glycoproteins Group (VGP) (Gifted)
Betacoronavirus	Sarbecovirus	Clade 1b	SARS-CoV-2	Wuhan-Hu-1 (19A)	Full Length Spike (FL-S)	Pirbright Institute - VGP (Gifted)
Betacoronavirus	Sarbecovirus	Clade 1b	SARS-CoV-2	Wuhan-Hu-1 (19A) Alpha, Delta, BA.1, BA.2, XBB, EG.5.1, BA.2.86, JN1	RBD	Pirbright Institute - VGP (Gifted)
Betacoronavirus	Sarbecovirus	Clade 1b	RaTG13 RacCS203	-	RBD	Pirbright Institute - VGP
Betacoronavirus	Sarbecovirus	Clade 2	RF1/2004 RS4081 RmYN02	-	RBD	Dr Jack Tan – University of Oxford (Gifted)
Betacoronavirus	Sarbecovirus	Clade 3	BtKY72	-	RBD	Dr Jack Tan – University of Oxford (Gifted)
Betacoronavirus	Sarbecovirus	Clade 5	Rc0319	-	RBD	Pirbright Institute - Viral Glycoproteins Group (Gifted)
Betacoronavirus	Merbecovirus	-	Middle East respiratory syndrome Coronavirus (MERS-CoV)	-	FL-S	Sinobio (40069-V08B) (Purchased)
Betacoronavirus	Embecovirus lineage	-	Human Coronavirus OC43 (HCoV-OC43)	-	FL-S	Addgene plasmid (R619-M66-303) (expressed, purified J.Chadwick)
Betacoronavirus	Embecovirus lineage	-	Human Coronavirus HKU1 (HCoV-HKU1)	-	FL-S	Sinobio (40606-V08B) (Purchased)
Gammacoronavirus	Igacovirus	-	Avian infectious bronchitis virus (IBV)	M41	FL-S	Pirbright Institute - Plasmid from Coronavirus Group (expressed, purified JC)
Betacoronavirus	Sarbecovirus	Clade 1b	SARS-CoV-2	Wuhan-Hu-1 (19A)	Nucleocapsid (N)	Cloned, expressed, purified J.Chadwick
Gammacoronavirus	Igacovirus	-	IBV	D388 and M41	N	Cloned, expressed, purified J.Chadwick
Henipavirus	-	-	Nipah	Bangladesh	Surface Glycoprotein (G)	Gifted by Nazia Thakur, VGP

Table 2.3: Recombinant viral proteins and their source.

All proteins were functionalized with HIS tag and purified by HisTrap column. Protein concentrations varied following purification and were diluted to the appropriate concentration according to the experiment as described in figure legends.

2.2 Amino acid alignments and phylogenetic trees

Sequences were derived from NCBI Genbank

(<https://www.ncbi.nlm.nih.gov/genbank>) (**Appendix 2**) and were aligned using DNASTar MegAlign Pro 7 (<https://www.dnastar.com/software/lasergene/megalign-pro/>) to determine amino acid sequence homology and generate phylogenetic trees.

2.3 Preparation of recombinant viral proteins

2.3.1 Cloning

SARS-CoV-2 whole genome nucleotide sequence was downloaded from NCBI Genbank (**Appendix 2**) and used for primer design as displayed in **Figure 2.1**. The expression vector PhL-SEC was selected for its compatibility with mammalian cell expression systems and included secretion signal sequence, Kozak sequences and a His tag region to facilitate protein purification. The vector was loaded into Snapgene and suitable restriction enzymes, Kpn1 and Age1, were identified for cloning. Primers were designed with the use of [NEBcloner](#).

A

Leader Sequence: (Spare Nucleotides)	Restriction site specific to Age1	Gene Insert (Stop codons removed – allow cell excretion)	Restriction site specific to Kpn1 enzyme	Spare Nucleotides
GCGC	ACCGGT	>NC_045512.2: 28274-29533	GGTACC	GCGC

B

Name	Length	Sequence	GC content	Mass
Primer 1 Pair 1 (Fwd)	35 nt (Age1)	aattaccggtATGTCTGATAATGGACCCCAAATC	40% GC	10,723.1 Da
Primer 2 Pair 1 (Rev)	35 nt (Kpn1)	aattggtaccGGCCTGAGTTGAGTCAGCACTGC	55% GC	10,169.7 Da
Primer 3 Pair 2 (Fwd)	28 nt (Age1)	aattaccggtATGTCTGATAATGGACCC	43% GC	8587.7 Da
Primer 4 Pair 2 (Rev)	28 nt (Kpn1)	aattggtaccGGCCTGAGTTGAGTCAGC	54% GC	8644.7 Da

Figure 2.1: Primer design for SARS-CoV-2 nucleocapsid cloning.

- A. Restriction site and insert details
 B. Primer sequences

A stock of SARS-CoV-2 RNA which had been reverse transcribed (Coronavirus Research Group, Pirbright Institute) was amplified by PCR with primers which flanked the N gene. PCR reactions were established using T4 polymerase (NEB #M0203S), 10 to 50 ng of cDNA template, Q5 reaction buffer (NEB #B9027S), 10 mM dNTPs and 10 µM of forward and reverse primer with nuclease free dH₂O added to a reaction volume of 50 µL. PCR was run on thermocycler (Applied Biosystems, Veriti). Following amplification, PCR products were run on 1% agarose DNA gel using SYBR Safe (Thermofisher Scientific #S33102) and a 1kb+ DNA ladder (Thermofisher Scientific #10787018), for 45min at 150V to check for the correct size fragment. Gels were visualised using UV gel dock.

The expression vector PhL-SEC was digested with Kpn1 and Age1 and run on a 1% agarose DNA gel as above to check for the correct size. Ligation was performed using

the New England Biolabs ligation protocol for T4 DNA ligase (#M0202). Vector and insert components were quantified by Thermofisher Scientific NanoDrop spectrophotometer and a molar ratio of 1:3 vector (0.02 pmol) to insert (0.06 pmol) used. All components excluding T4 DNA ligase were combined and stored on ice before mixing. Ligase was added at room temperature for 10 minutes before heat inactivation for 10 minutes at 65°C.

Resulting recombinant plasmids (1.5×10^{-3} µg) were added to *E.coli* cells (Oneshot MAX efficiency DH5-Alpha, Thermofisher Scientific #18258012) incubated on ice for 20 minutes, 42°C water bath for exactly 30 seconds then back to ice for 5 mins. SOC media (250 µL) (Thermofisher Scientific #15544034) was added and incubated at 37°C for 1hr in a shaking incubator. Transformed cells were spread onto agar ampicillin selection plates and incubated overnight inverted at 37°C. Single colonies were picked and transferred to conical flasks for 14hrs. Following this, cells were pelleted by centrifugation at 20k RPM before storage at -20°C.

Plasmid was purified from stored *E. coli* cell pellets by QIAGEN maxiprep kit (#12162). Manufacturers instructions were followed. DNA was quantified by NanoDrop, and Sanger sequenced.

2.3.2 Protein expression and purification

Recombinant viral structural proteins were expressed via the mammalian cell expression system Expi293 (ExpiFectamine™ 293 Transfection Kit, Thermofisher #A14524). Human derived Expi293 competent cells were cultured directly in the Expi293™ Expression Medium to a high cell density in suspension (6×10^6 cells/g). DNA plasmid (30 µg) encoding the protein of interest was combined with PEI max transfection reagent (Polysciences #24765-1) in reduced-serum medium Opti-MEM™ and added to 30mL cell suspension. Expi293 Transfection Enhancers (300 mM

valproic acid, 500 mM sodium propanoate and 2.5M of glucose) were added to the culture 20 hours post-transfection. Following transfection and enhancer addition, the cells were transferred back to shaking incubator in 37°C 5% CO₂ for 5 to 7 days, during which the recombinant protein was expressed and secreted into the culture medium. Cell supernatant was harvested by centrifugation at 4750 rpm for 20 minutes.

Cell supernatant was equilibrated to 20 mM NaPO, 0.5 mM NaCl and 20 mM imidazole before purification using the functional His tag affinity chromatography via Cytiva HisTrap High Performance 5 mL columns (Cytiva #17524802). The capture column was flushed with H₂O, then binding buffer (20 mM NaPO, 0.5 mM NaCl and 20 mM imidazole, pH 7.4) for 5x column volumes before addition of cell supernatant. Captured recombinant protein was eluted using a gradient of buffer with increasing imidazole concentrations from 62.5 mM-1000 mM. Each concentration used a total volume of 4 mL which was collected as 2x 2 mL protein fractions. Residual imidazole present in protein fractions, was removed by Zeba™ Spin Desalting Columns (ThermoFisher Scientific #89883).

Evaluating the mass of recombinant proteins was performed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad 12 well 20% polyacrylamide Mini-PROTEAN® TGX™ gels (#45610450). Purified protein samples were combined 1:3 with 4x Laem mLi sample buffer (Bio-Rad #1610737) containing SDS, β-mercaptoethanol and tracking dye followed by heating to 80°C for 10 minutes. A pre-stained molecular weight ladder (Bio-Rad precision plus #1610374) was used. Electrophoresis was carried out in Tris-Glycine-SDS running buffer, at 150V. To visualise protein bands, gels were stained with Coomassie Brilliant Blue R-250 staining solution. Following destaining, gels were placed on Bio-Rad gel dock where imaging was performed in Image studio 5.2.

Western blotting was carried out by semidry transfer onto nitrocellulose membrane, followed by incubation in blocking solution, 0.5% milk powder in PBS containing 0.1% Tween-20 (PBS-T). Primary antibody, anti-His tag mAb (Invitrogen #MA1-21315) was diluted 1/1000 in blocking solution and applied to membranes for 1 hour at room temperature before washing with 0.1% PBS-T. IRDye® 800CW goat anti-mouse IgG secondary antibody (LI-COR, #926-32210) was diluted 1/15,000 in blocking solution and applied to membranes for 1 hour. Membranes were washed with 0.1% PBS-T at room temperature before visualisation on a LICOR Odyssey Scanner.

Recombinant protein concentrations were determined by NanoDrop in the A280 mode, using defined extinction coefficient (ϵ) and molecular weight (MW) for accurate measurements. Instrument calibration and blanking were conducted with the same buffer used for final protein elution (PBS). The theoretical extinction coefficient and molecular weight (MW, in Da) of the protein were calculated using the ProtParam tool, based on the amino acid sequence. The molecular weight was likewise estimated from the full-length amino acid sequence, including any affinity tags or linker peptides.

2.4 ELONA development

2.4.1 Checkerboard optimisation of coating antigen and monoclonal antibody

SARS-CoV-2 spike protein was diluted in carbonate buffer and immobilised on Nunc-Immuno MaxiSorp™ 96-well plates (Thermofisher #161093) in the range of 50 μ L/well at 0.1 μ g/mL (5 ng/well) to 12.5 μ g/mL (625 ng/well overnight). Wells were blocked with 50 μ L/well of 2% BSA, followed by a range of dilutions of the primary mAb, CR3022 (Viral Glycoproteins Group) at 50 μ L/well at 0.05 μ g/mL (2.5 ng/well) to 50 μ g/mL (2.5 μ g/well). The secondary antibody was anti-human IgG-horseradish peroxidase (Cell Signaling Technology #32935) added at 50 μ L/well as a 1/1000 dilution. Plates were washed with 0.05% Tween-20 between each step. Following

incubation with 100 μ L/well of the substrate 3,3',5,5'-tetramethylbenzidine (Ultra TMB, Thermofisher Scientific #34028) for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm.

2.4.2 Enzyme-Linked Oligonucleotide Assay format and optimisation.

In the developed ELONA format, flat bottom Nunc-Immuno MaxiSorp™ 96-well plates were coated with 50 μ L of 1.6 μ g/mL target antigen (80 nG/well) diluted in carbonate buffer (Sigma Aldrich #C3041) and incubated overnight at 4°C. Wells were blocked with 50 μ L/well of 10 μ M Z-Block (SomaLogic) stock in 2% BSA, 0.1% Tween-20, 10 mM MgCl₂+, PBS for 1hr at room temperature. Blocking reagent was removed by flicking plates before washing with 100 μ L of 0.5% PBS-Tween-20 three times before the plate was tapped dry.

Biotin conjugated SOMAmer (50 μ L/well) of 10 nM stock or anti-His tag positive control mAb (Thermofisher Scientific #21315) at 1 μ g/mL in Z-block mix was added to wells and incubated for 1 hour at room temperature. Plates were washed again three times with 0.5% PBS-Tween-20 and tapped dry. Ultra Streptavidin-horseradish peroxidase (Thermofisher Scientific #N504) was diluted 1/1600 from stock in Z-block mix before 50 μ L/well applied to SOMAmer and secondary only wells. Alternatively, anti-mouse IgG, HRP linked (Cell Signaling Technology #7076S) was diluted 1/1000 from stock and 50 μ L/well applied to anti-His mAb wells. Secondaries were incubated for 1hr at room temperature.

Plates were washed three times with 100 μ L of 0.5% PBS-Tween-20 a total of 3 and tapped dry. TMB Ultra solution at 100 μ L/well was added and allowed to develop for 5mins at room temperature before being quenched by 1 M sulphuric acid solution (Merck, #258105). Optical density (OD) of wells was measured at 450 nM using a plate reader.

Raw OD values were expressed as a proportion of the anti-His tag antibody positive control as a signal/positive ratio as calculated below . The signal threshold was set at S/P 0.2, with readings <0.2 considered negative [81].

Calculation of S/P ratio

$$\frac{\text{Mean of Test Sample} - \text{Mean of negative control}}{\text{Mean of Positive control} - \text{mean of negative control}} = S/P$$

Optimisation experiments included investigating parameters such as coating antigen levels, different blocking agents, different sources and concentrations of streptavidin-HRP and washing buffers as explained in **Chapter 3**. Alternative blocking agents included Seablock (Thermofisher Scientific #37527), BSA and deoxynucleotide triphosphates (dNTPs). Sources of streptavidin-HRP were S911 (Thermofisher Scientific #S911) and Ultra Strep (as above).

2.5 Epitope competition analysis

2.5.1 Competition ELONA

The developed ELONA method was used as described above apart from an additional step after blocking, where amine-version SOMAmers were applied to wells at 50 µL/well of 30 nM to occupy epitopes. Following washing steps, biotin tagged SOMAmers were added and the ELONA completed as described above.

2.5.2 Carterra LSA Surface Plasmon Resonance analysis – classical epitope binning and kinetics

Firstly, the chip was prepared by preconditioning with 60 second cycles of 50 mM NaOH, 1 M NaCl and 10 mM glycine at pH2. SOMAmer reagents were refolded at 1µM before capture. Oligonucleotides were heated at 95°C for 15 minutes in a thermocycler and cooled for 20 minutes at room temperature before dilution in SOMAmer capture and buffer (5 mM MgCl₂, 40 mM HEPES, 0.05% Tween-20). Biotinylated SOMAmers were immobilised onto flow chips SAHC30M Streptavidin

(Carterra #4294) (polycarboxylate hydrogel with medium charge density and 30 nM coating thickness).

Ligand capture at concentrations of 1000 nM, 100 nM and 10 nM biotin tagged SOMAmers in triplicate were performed over 10 minutes. Following capture, the chip sensor was washed with regeneration buffer for 2x30 seconds. Loading of SOMAmers was observed with typical capture at 10 nM of 400 response units (RU), typical capture at 100 nM of 750 RU, and a typical capture at 1000 nM of 1100 RU which was marked as sufficient for reporting kinetic parameters.

Following capture of SOMAmers, the running buffer for binning changed to SB17, 0.05% Tween-20. The sensor was then exposed to the viral antigen diluted in SB17 (containing 20µM Z-block) to a protein concentration of 1.5 µg/mL or 60 nM and loaded for 7 minutes of injection and cycling over immobilised SOMAmers. The RU measurement for antigen association with capture SOMAmer established a baseline for determining sandwich binding or blocking RU response by amine SOMAmers.

Amine SOMAmer (1 µM) was injected over the capture locations as a binding competitor to biotinylated SOMAmers, binding to the bound antigen at a range of viral protein concentrations between 20 nM to 100 nM.

Following completion of the binning cycle with antigen and amine were removed using 2 x 30 second pulses of regeneration buffer (25 mM NaOH and 1 M NaCl). SAHC30 surface SOMAmers were regenerated and combined with further viral antigens. Interpretations of epitope groupings were produced by Carterra LSA analysis software. New flow chips SAHC30M Streptavidin were used for kinetics analysis. SOMAmers were diluted in a running buffer (SB17, 0.05% Tween-20, 20 µM Z-block) to 8 concentrations in a fivefold dilution series between 1000 nM and 0.0128 nM. SOMAmer concentrations below 8 nM did not provide binding data. Kinetics

measurements were run in SB17 buffer not containing Z-Block using 5 minutes association time and 15 minutes dissociation, regeneration injections between antigens (multi-cycle kinetics) using regeneration buffer as above. Interpretations of kinetics data were produced by Catterra LSA analysis software.

2.6 LFT prototype development

2.6.1 Gold nanoparticle labelling of detection SOMAmers.

Negatively charged 5'-thiol SOMAmers were attached to gold nanoparticles (AuNPs) via a method developed by Mengqi et al, 2022 and modified by EDX Medical (proprietary information). AuNP was obtained from DCN Diagnostics (#CG010).

2.6.2 Prototype development

Optimisation studies varied the experimental design as described in **Chapter 5**, with common components which included the following.

Prototypes were constructed using sample pad (Cytiva #CF4), conjugate pad (Cytiva #ST20), nitrocellulose membrane (Cytiva #FF80-HP), and wicking pad (Cytiva #CF3).

The recombinant viral antigen used was SARS-CoV-2 Wuhan Hu-1 RBD (Sinobio #40592-V08B), either desiccated onto nitrocellulose (2ng), diluted in running buffer (1.5 µg in 150 µL) or pre-combined with detection SOMAmers.

Running buffer was used to dilute RBD and SOMAmers before running on nitrocellulose membranes, PBS, 4.5% BSA, 100 µM Z-block, 139 mM MgCl₂ in PBS. Detection Au-NP SOMAmers were diluted to an OD of 2 or 8 in a final volume of 100 µL or dried onto the conjugate pad. Capture 5'-SENPAH SOMAmers were desiccated onto the nitrocellulose membrane deposited either by pipetting (750 µL) or Biodot machine, which provided rows of five (6 nL) dots positioned with 325 nM between horizontal locations and 1 mm between vertical rows.

Chapter 3: Enzyme-linked oligonucleotide assay development

3.1 Introduction

This chapter describes the preparation of materials and screening methods to analyse the viral antigen-binding capacity of a custom SOMAmer library for potential use as molecular recognition elements in diagnostic applications, e.g. as countermeasures for emerging pandemic threats.

A library of 45 SOMAmers selected against the SARS-CoV-2 Wuhan-Hu-1 (19A) spike protein was provided by SomaLogic for these studies. In addition, one SOMAmer selected against SARS-CoV-2 Wuhan-Hu-1 nucleocapsid and two SOMAmers selected against SARS-CoV nucleocapsid were available and used as control reagents.

3.1.1 The use of Enzyme Linked Oligonucleotide Assay for candidate screening

To provide scalability, an ELONA assay format was chosen to screen the library of candidate SOMAmers, allowing multiple SOMAmers to be evaluated for binding against multiple antigens, reducing experimental time and cost. Similar to ELISA, ELONA has numerous elements each requiring optimisation, including coating antigen, reagent concentrations, blocking and washing procedures.

Successful ELONA requires bipartite interactions between viral antigen and nucleic acid molecules, in this case SOMAmers. This is a step towards the tripartite interactions required in an LFT format where capture-antigen-detection formations are required.

3.1.2 Investigating specificity in SOMAmer-based ELONA

In developing the ELONA, it was necessary to establish assay conditions that showed specific binding between SOMAmers and their SELEX target. The developed assay could then be taken forward to further experiments to investigate the breadth of binding to divergent coronaviruses. The provided SOMAmer library had not been used in ELONA experiments previously, therefore there was no known SOMAmer which bound to SARS-CoV-2 RBD/S1/S2 available as a positive control at this stage.

To control for non-specific antigen binding, SOMAmers were exposed in ELONA to SARS-CoV-2 proteins not used for SELEX process, or to unrelated viral antigens. For example, SOMAmers derived from SELEX processes using SARS-CoV and SARS-CoV-2 nucleocapsid were compared with SOMAmers generated against SARS-CoV-2 Wuhan-Hu-1 spike binding to spike and nucleocapsid protein respectively.

3.1.3 Expression of viral structural proteins

A starting point for assay development involved the cloning, expression and purification of recombinant coronavirus antigens for coating ELONA assay plates. As the SOMAmer library was selected with SARS-CoV-2 Wuhan-Hu-1 RBD/S1/S2, production of SARS-CoV-2 full length spike protein was a key reagent. For controls to investigate non-specific SOMAmer binding, SARS-CoV-2 Wuhan-Hu-1 nucleoprotein and OC43 (human seasonal Beta-CoV) full length spike protein were also produced. Other recombinant viral plasmids/proteins were available from lab stocks and included SARS-CoV-2 Wuhan-Hu-1 RBD, Infectious Bronchitis virus (avian respiratory Gamma-CoV) strains D388 and M41 nucleocapsid, and Nipah Bangladesh surface glycoprotein. Examples of viral structural protein production are shown below.

3.2 Results

3.2.1 Generation of Coronavirus protein expression constructs and protein purification

3.2.1.1 Cloning, expression and purification of SARS-CoV-2 nucleocapsid

As an example, the plan for production of the recombinant plasmid for the expression of SARS-CoV-2 nucleocapsid is shown in **Figure 3.1**.

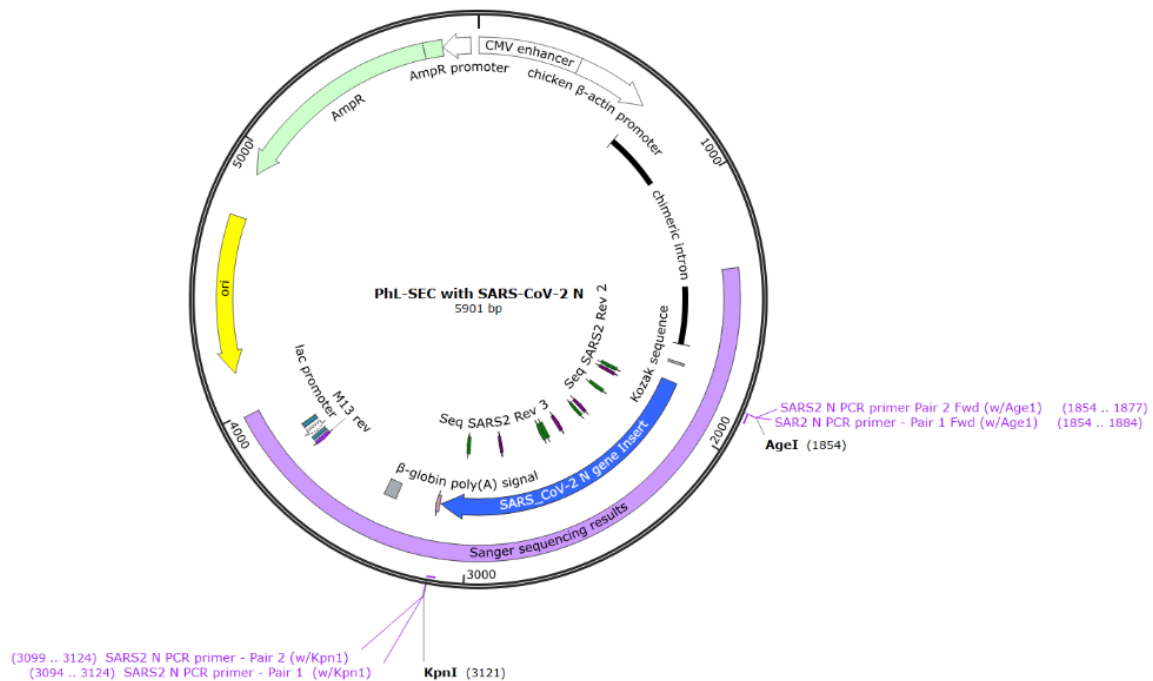


Figure 3.2: A SARS-CoV-2 nucleocapsid expression plasmid.

Expression plasmid with SARS-CoV-2 nucleocapsid protein coding region inserted into the vector backbone PhL-SEC provided a compatible promoter system for the mammalian expression system Expi293, with Kozak sequences and His-tag used for purification and validation. Location of the insert between restriction cloning sites Age1 and Kpn1 is illustrated, in addition to the location of primers used for Sanger sequencing.

The 1.2kb target nucleoprotein gene insert was generated by PCR (**Figure 3.2**). Vector was digested, and the correct size fragments of vector (4.6kb) and insert (1.2kb) prior to the ligation step were demonstrated (**Figure 3.2A**). Following ligation and transformation into *E.coli*, plasmid preparations from individual colony growths were digested and run on a DNA gel (**Figure 3.2B**) to confirm the correct size fragments for

the vector and nucleocapsid gene insert. In addition, the expression plasmid was examined by Sanger sequencing to ensure the correct sequence.

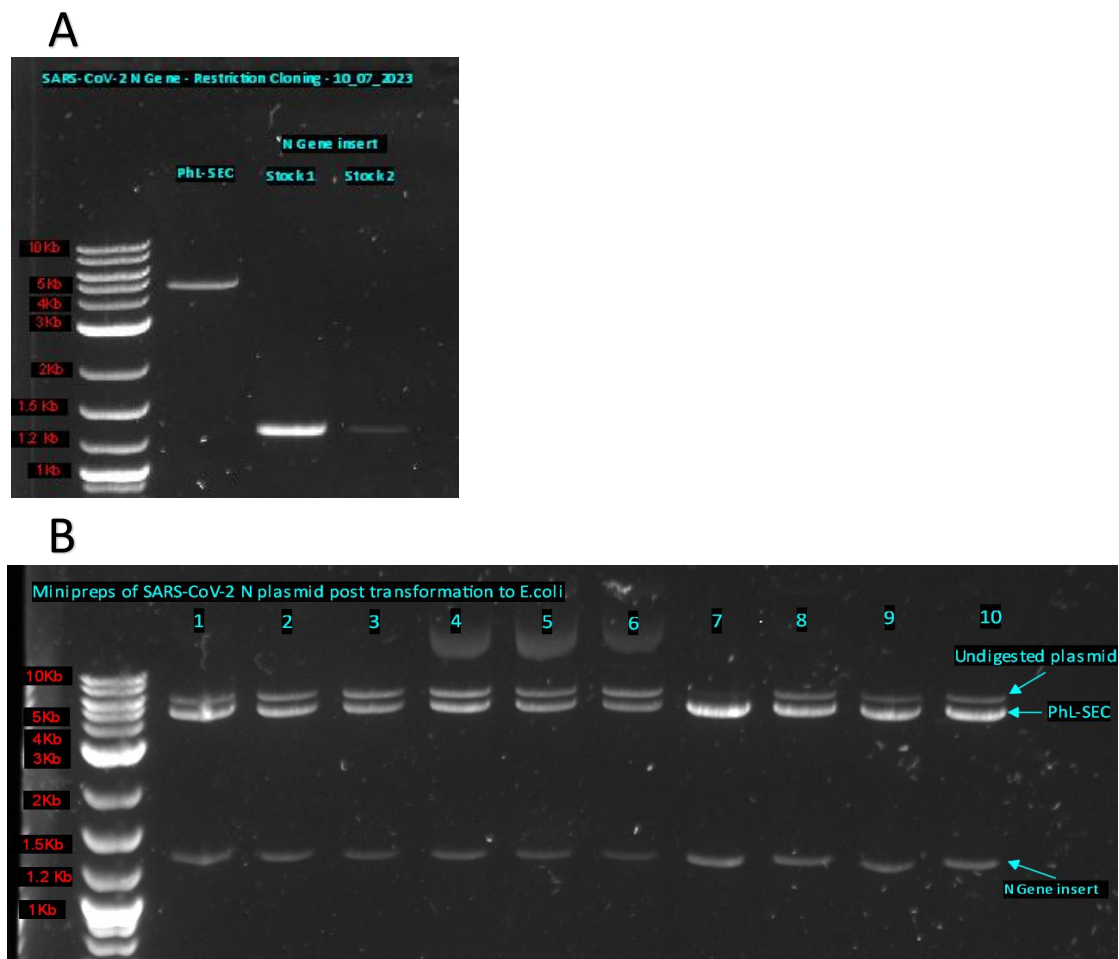


Figure 3.2: Generation of SARS-CoV-2 nucleoprotein expression plasmid.

A. 1% DNA gel of expression plasmid components pre-ligation as shown in Figure 3.1, PhL-SEC linearised vector backbone, and PCR product of SARS-CoV-2 nucleocapsid gene insert (Stock 1 and Stock 2). Molecular weights are shown against the NEB 1 kb Plus DNA Ladder.

B. 1% DNA gel demonstrating plasmid preparations from *E.coli* transformed with PhL-SEC containing the SARS-CoV-2 N gene (Columns 1-10). Plasmid was digested with Age1-HF and Kpn1-HF showing the expected vector backbone, insert and undigested plasmid bands. Molecular weights are shown against the NEB 1 kb Plus DNA Ladder.

Plasmid stocks were transfected into the eukaryotic cell system for protein expression and purification (**Figure 3.3A and B**). Collected fractions showed protein of the correct molecular weight (46 kDa) by Coomassie Blue staining (**Figure 3.3A**) which was further confirmed by Western blot (**Figure 3.3B**). Briefly, expression plasmids encoding SARS-CoV-2 nucleocapsid were transfected into the Expi293 mammalian cell expression system, and cell supernatant harvested. A series of elution fractions using imidazole as a binding competitor for Ni⁺ ions present in the column enabled protein purification. Fractions were then run on an SDS PAGE protein gel and stained with Coomassie Blue. The high concentrations of target protein were identified by the most abundant bands of the correct molecular weight (46 kDa) prior to being pooled and buffer exchanged to generate recombinant protein stocks. Anti-His tag mAb was used in Western Blotting to demonstrate purified nucleoprotein of the correct molecular weight (**Figure 3.3B**).

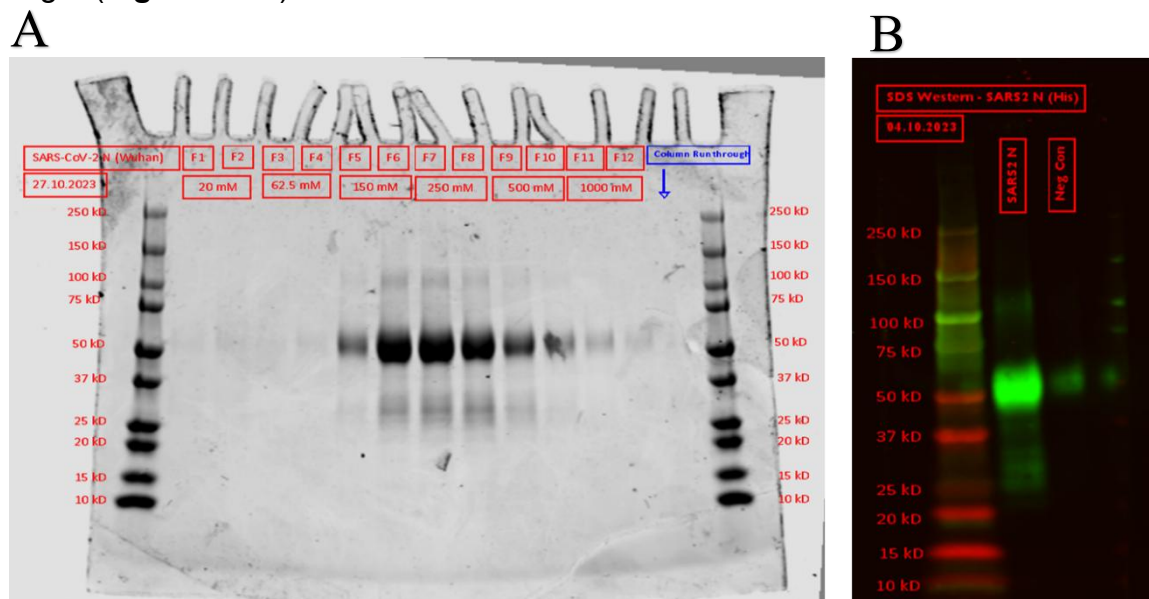


Figure 3.3: Purification and Western Blot of SARS-CoV-2 nucleoprotein

A. Following transfection of Expi293 cells with the expression plasmid for SARS-CoV-2 Wuhan-Hu-1 nucleocapsid (as shown in Figure 3.1), supernatant was collected and protein captured on a His-trap column. Protein was eluted with increasing mM concentrations of imidazole and fractions run on an SDS PAGE gel followed by staining with Coomassie Blue. Collected fractions 5 to 10 show protein of the correct molecular weight (MW) run against Precision Plus Protein Dual Colour Standards.

B. Fractions 6 to 8 were pooled, run on an SDS PAGE and Western blotted with anti-His mAb labelled with a green fluorochrome, confirming correct molecular weight.

3.2.1.2 Expression and purification of SARS-CoV-2 Wuhan-Hu-1 full length spike.

For assay development, the reference viral protein used in the SOMAmer library selection was expressed and purified. SARS-CoV-2 Wuhan-Hu-1 full length spike expression plasmid was obtained from Addgene, and purified protein was produced in an identical process to SARS-CoV-2 nucleocapsid above. Collected fractions showed protein of the correct molecular weight (180 kDa) by Coomassie Blue staining (**Figure 3.4A**) which was further confirmed by Western Blot (**Figure 3.4B**).

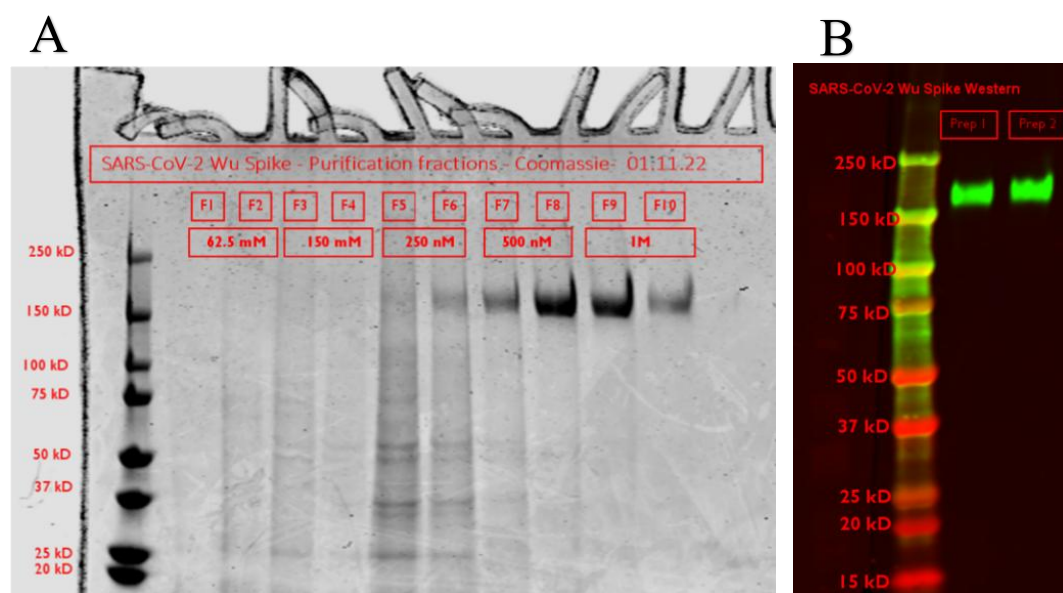


Figure 3.4: Purification and Western Blot of SARS-CoV-2 full length spike

A. Following transfection of Expi293 cells with the expression plasmid for SARS-CoV-2 Wuhan-Hu-1 spike protein, supernatant was collected and protein captured on a His-trap column. Protein was eluted with increasing mM concentrations of imidazole and fractions run on an SDS PAGE gel followed by staining with Coomassie Blue. Collected fractions 7 to 10 show protein of correct molecular weight (MW) run against Precision Plus Protein Dual Colour Standards.

B. Fractions 7 to 10 were pooled, run on an SDS PAGE and Western blotted with anti-His mAb labelled with a green fluorochrome, confirming correct molecular weight.

3.2.1.3 Expression and purification of OC43 full length spike.

As a non-SARS-CoV-2 control, the spike protein of OC43 was expressed recombinantly from a commercially available expression plasmid from Addgene. Identical to the expression of other proteins, post purification fractions were run on SDS PAGE and stained with Coomassie Blue (**Figure 3.5**), before collection of fractions, buffer exchange and quantification. Collected fractions showed protein of the correct molecular weight (148 kDa) by Coomassie Blue staining.



Figure 3.5: Purification of OC43 full length spike

OC43 spike protein was eluted with increasing mM concentrations of imidazole and fractions run on an SDS PAGE gel followed by staining with Coomassie Blue. Collected fractions 5 to 8 show protein of correct molecular weight (mw) against Precision Plus Protein Dual Colour Standards.

3.2.1.4 Summary of recombinant coronavirus proteins used for ELONA development.

Recombinant proteins used in ELONA development are shown in **Table 3.1**.

Virus species	Variant	Structural Protein	Source
SARS-CoV-2	Wuhan-Hu-1 (19A)	Full Length Spike (FL-S)	Expressed, purified J.Chadwick
SARS-CoV-2	Wuhan-Hu-1 (19A)	Nucleocapsid (N)	Cloned, expressed, purified J.Chadwick
SARS-CoV-2	Wuhan-Hu-1 (19A)	Receptor Binding Domain (RBD)	Gifted by Joe Newman, Viral Glycoproteins Group
OC43	-	Full Length Spike (FL-S)	Expressed, purified J.Chadwick
IBV	D388 and M41	Nucleocapsid (N)	Cloned, expressed, purified J.Chadwick
Nipah	Bangladesh	Surface Glycoprotein (G)	Gifted by Nazia Thakur, Viral Glycoproteins Group

Table 3.1: A summary of the recombinant coronavirus proteins used in development of the ELONA assay.

All proteins were functionalized with HIS tag and purified by HisTrap column. Protein concentrations varied following purification and were diluted to the appropriate concentration according to the experiment as described in figure legends.

3.2.2 Establishing the ELONA format and optimisation of viral antigen coating levels.

The ELONA design for assessment of SOMAmer binding and positive controls is demonstrated in the immunostaining plan shown in **Figure 3.6**. Viral antigen (shown here as spike protein) was coated onto plastic wells and subsequently incubated with blocking agent. Biotin-tagged SOMAmers were added to allow binding to antigen. Subsequently, streptavidin-HRP conjugate was incubated to allow binding to the biotin tag, and the presence of bound HRP revealed by conversion of TMB to a signal read at an absorbance of 450 nm.

A positive control was based on the His tag present on all recombinant proteins used as plate coating antigens. His tag specific mAb was bound to coated antigen, followed

by species specific secondary mAb conjugated to HRP. Again, the presence of bound HRP was measured by conversion of TMB to a signal read at an absorbance of 450 nm. For some ELONA development experiments using SARS-CoV-2 spike coating antigen, the specific mAb CR3022 was used as positive control in place of His tag specific mAb. The mAb CR3022 [113] was generated against SARS-CoV RBD but also binds SARS-CoV-2 RBD.

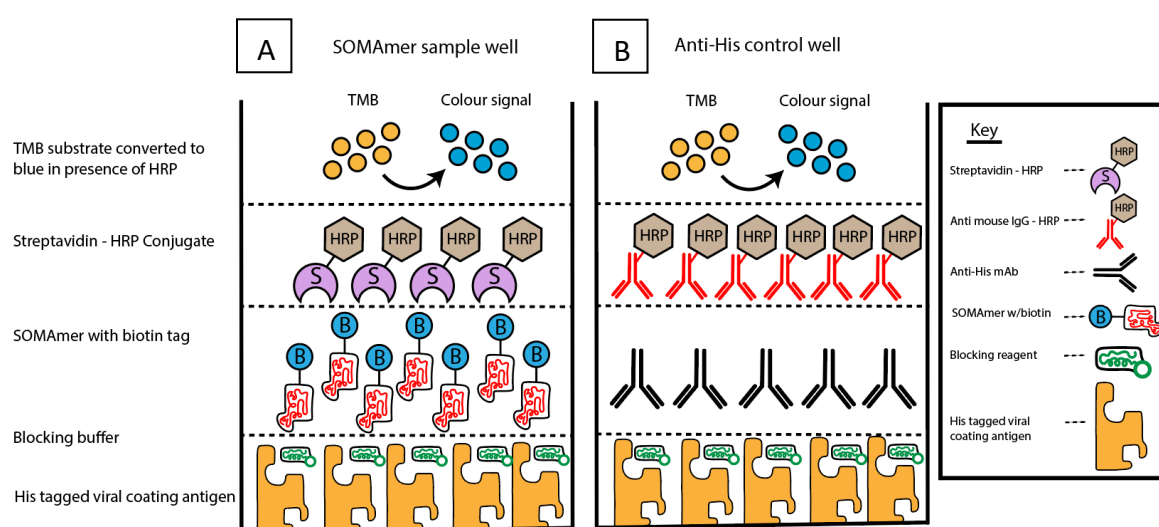


Figure 3.6: Schematic of ELONA for SOMAmer evaluation.

- His-tagged recombinant viral antigen was coated onto wells, and blocking reagent added. Biotin-tagged SOMAmers were allowed to bind to the viral antigen. Streptavidin: HRP was added to allow binding to biotin groups. HRP presence was visualised by adding TMB substrate followed by reading of absorbance at 450 nm.
- As the control well, His-tagged viral antigen was bound by anti-His mAb, followed by species-specific mAb:HRP conjugate. HRP presence was visualised by adding TMB substrate followed by reading of absorbance at 450 nm.

A checkerboard format was used to establish optimal concentrations of both SARS-CoV-2 spike protein as plate coating antigen and positive control anti-SARS-CoV RBD mAb (CR3022) which generated signal values between 1 and 2 (OD450) (**Figure 3.7**). The concentration of coating viral antigen used for subsequent screening studies was 1.6 µg/mL (80 nG/well) which provided a binding signal within the range of 1 to 1.5 at

OD450 (**Figure 3.7B**). The chosen concentration of mAb CR3022 for future experiments was 1.6 $\mu\text{g}/\text{mL}$ (80 nG/well).

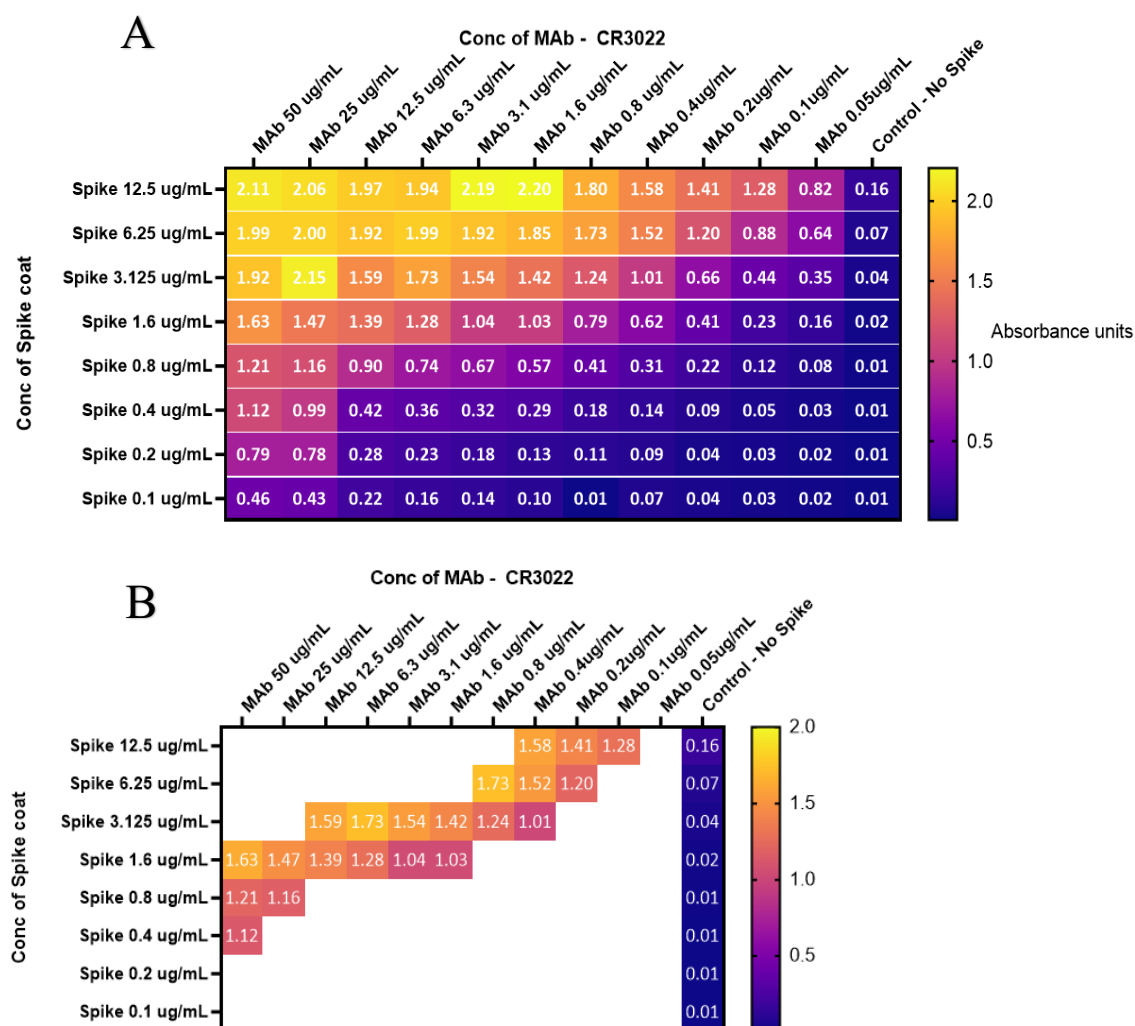


Figure 3.7: SARS-CoV-2 spike ELISA checkerboard titration to establish appropriate concentrations of coating antigen and anti-RBD monoclonal antibody (CR3022).

A. ELISA conditions included; the coating antigen was 50 $\mu\text{L}/\text{well}$ of SARS-CoV-2 spike in the range of 12.5 $\mu\text{g}/\text{mL}$ (625 ng/well) to 0.1 $\mu\text{g}/\text{mL}$ (5 ng per well); 50 $\mu\text{L}/\text{well}$ of mAb CR3022 was added in the range of 50 $\mu\text{g}/\text{mL}$ (2.5 $\mu\text{g}/\text{well}$) to 0.05 $\mu\text{g}/\text{mL}$ (2.5 ng/well); the blocking agent was 50 $\mu\text{L}/\text{well}$ of 2% BSA; 50 $\mu\text{L}/\text{well}$ of 1/1000 stock of anti-mouse IgG-HRP was used as a secondary antibody; the washing buffer between each step was 0.05% Tween-20 in PBS. Following incubation with 100 $\mu\text{L}/\text{well}$ of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. The colour intensity on the heatmap corresponds to the binding signal.

An additional positive control utilised the His tag present on each of the coating viral antigens. Anti-His tag mAb was titrated against 80 ng/well of SARS-CoV-2 spike protein, and the concentration of 0.25 $\mu\text{g/mL}$ (12.5 ng/well) which resulted in an absorbance value range of 1.3 to 1.5 at OD450 was selected for future experiments (**Figure 3.8**). This positive control verified adequate viral antigen coating was present in assay wells.

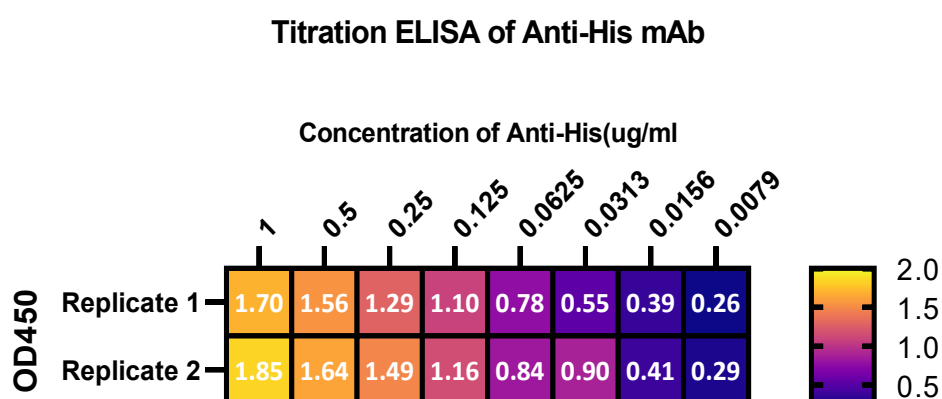


Figure 3.8: SARS-CoV-2 spike ELISA checkerboard titration to establish an appropriate concentration of anti-His monoclonal antibody.

ELISA conditions included; the coating antigen was 80 ng/well of SARS-CoV-2 spike; 50 μL /well of anti-His mAb was added in the range of 1 $\mu\text{g/mL}$ (50 ng/well) to 0.0079 $\mu\text{g/mL}$ (0.4 ng/well); the blocking agent was 50 μL /well of 2% BSA; 50 μL /well of 1/1000 stock of anti-mouse IgG-HRP was used as a secondary antibody; the washing buffer between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μL /well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. The colour intensity on the heatmap corresponds to the binding signal.

3.2.3 Establishing the ability of SOMAmers to bind viral antigen in pilot ELONA format.

At this initial stage, it was not known if SOMAmers were capable of binding to expressed viral proteins in the ELONA format. Therefore, a pilot experiment where SARS-CoV-2 selected SOMAmers were first used in the ELONA format (as displayed in **Figure 3.6**) was established to determine if binding and detection could be achieved.

In addition to SARS-CoV-2, the viral strain used for selection of the SOMAmer library, the spike protein of a betacoronavirus, OC43, was also examined for SOMAmer

binding as an unrelated antigen control (**Figure 3.9**). The SOMAmer library showed binding to SARS-CoV-2 spike, but also to OC43 spike. Prior to the establishment of alternative controls (such as nucleocapsid) in further ELONA assay development, high binding response by all SOMAmer candidates to OC43 spike, which has limited amino acid identity to Wuhan-Hu-1 spike (38%), suggested the presence of non-specific binding.

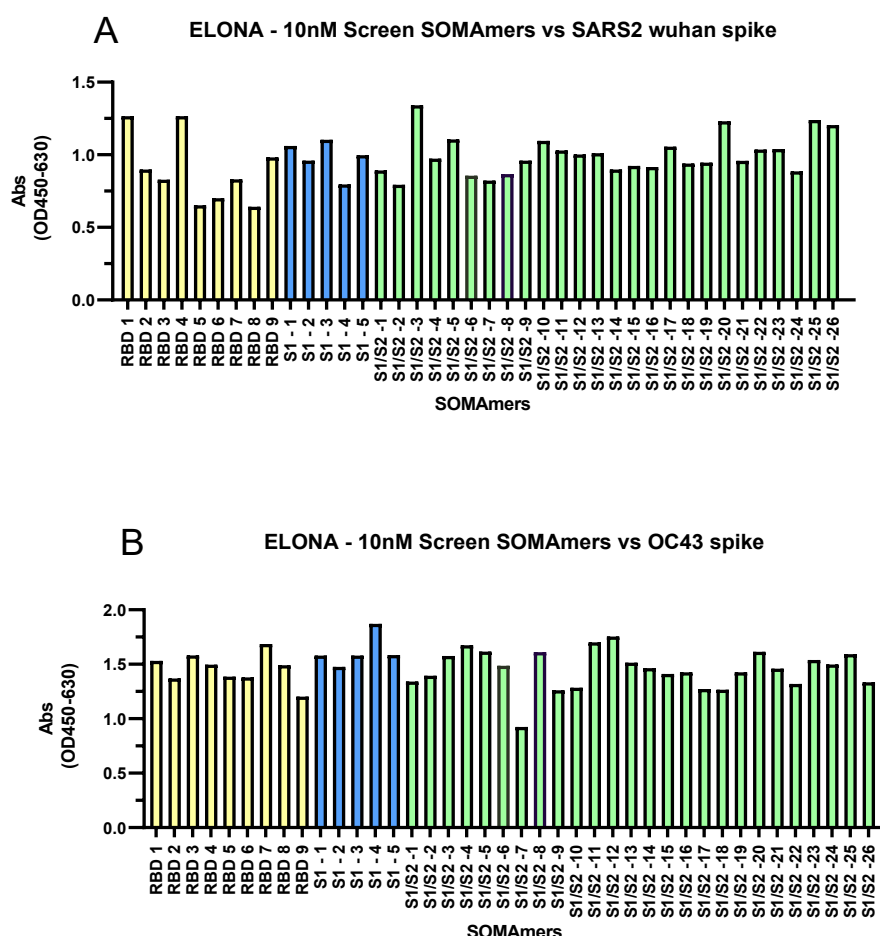


Figure 3.9: Pilot ELONA evaluating SARS-CoV-2 spike selected SOMAmers binding to SARS-CoV-2 and OC43 spike proteins.

The ELONA format was as described in Figure 3.6, conditions included; the viral coating antigen (80 ng per well) was either SARS-CoV-2 Wuhan-Hu-1 spike (panel A) and OC43 spike (panel B); library SOMAmers selected against RBD, S1 and S1/S2 were applied at 50 μ L/well of 10 nM stock; the blocking agent was 50 μ L of 2% BSA; streptavidin-HRP at 50 μ L/well of 1/400 dilution of 1mg/mL stock was added; washing buffer between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L/well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. Binding response data averaged from triplicate technical repeats and presented as raw OD signal.

Following demonstration of potentially non-specific binding by SOMAmers, further investigations were carried out to investigate binding specificity. A wider range of target viral proteins, including SARS-CoV-2 spike, SARS-CoV-2 nucleocapsid (N), OC43

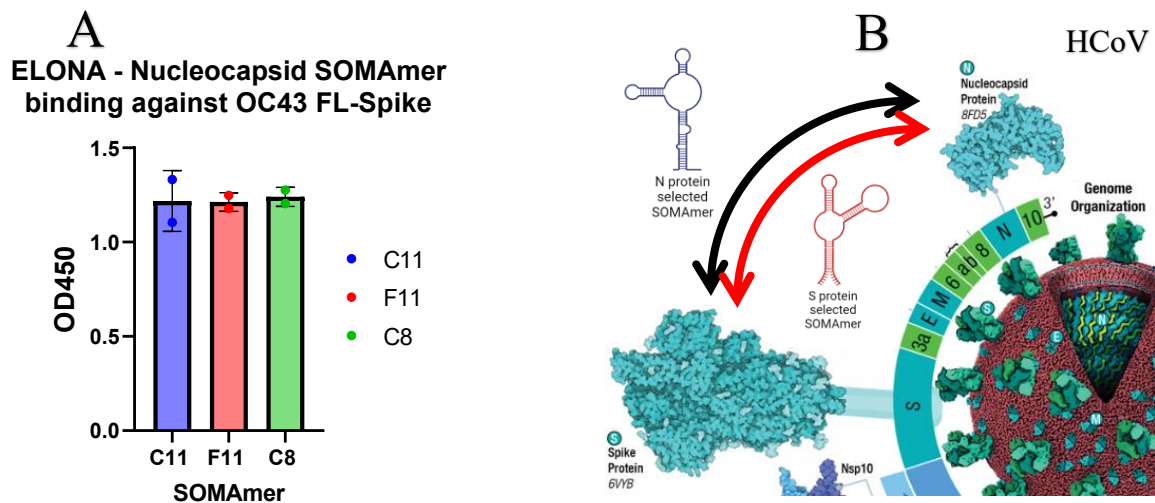


Figure 3.11: Further confirmation of non-specific interaction between N SOMAmers and OC43 spike

A. The ELONA format was as described in Figure 3.6, conditions included: the viral coating antigen (80 ng/well) was control antigen for non-specific binding OC43 spike; SARS-CoV-2 and SARS-CoV nucleocapsid SOMAmers (C11, F11, C8) were applied at 50 μ L/well of 10 nM stock; the blocking agent was 50 μ L of 2% BSA; streptavidin-HRP was added at 50 μ L/well of 1/400 dilution of 1 mg/mL stock; the washing buffer between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. SOMAmers had not been selected in SELEX by OC43 Spike.

B. Schematic representing non-specific interactions. OC43 spike represented a protein structure not used in the SARS-CoV and SARS-CoV-2 N SOMAmers SELEX generation but displayed non-specific binding in ELONA. Illustrated in red (spike SOMAmer) and black (N SOMAmer) are non-specific interactions observed in Figures 3.10 and 3.11A. Binding response data averaged from triplicate technical repeats and presented as raw OD signal.

Further evidence of non-specific binding interaction was shown by SARS-CoV N and SARS-CoV-2 N selected SOMAmers in a repeat study which displayed binding to OC43 spike, a protein structure not used in the generation of these SOMAmers (**Figure 3.11A**). SOMAmers selected against N, whether SARS-CoV or SARS-CoV-2 would not be expected to bind SARS-CoV-2 spike or OC43 spike, or Nipah G as there is a low sequence identity between targets. Similarly, SOMAmers selected against SARS-CoV-2 spike, whether RBD, S1 or S1/S2, would not be expected to bind SARS-CoV-2 N, neither IBV N nor Nipah G as there is minimal sequence identity. As a result,

this data indicated that non-specific interaction was occurring within the assay, potentially as the result of SOMAmers having non-specific binding to non-target antigens despite the presence of 2% BSA as a blocking agent. This therefore suggested that enhanced blocking of antigens would be the first area examined in ELONA optimisation.

3.2.4 ELONA Optimisation: reducing non-specific binding

To investigate the effect of different blocking reagents, a subset of SOMAmers derived against separate SELEX antigens (RBD, S1, S1/S2, and SARS-CoV/SARS-CoV-2 N) were used. These SOMAmers were assayed against three viral antigen targets, SARS-CoV-2 spike, OC43 spike and IBV N. The desired outcome was demonstration of specific binding of SOMAmers to their homologous SELEX target, and reduced binding of SOMAmers to targets unrelated to their SELEX procedure.

Hydrophobic patches or charged domains are known regions of interaction of SOMAmers preventing non-specific binding if effectively blocked. Within ELISA and ELONA, blocking reagents act as inert space fillers ensuring that MREs do not bind to available target surfaces non-specifically.

The blocking agent used up to this stage was 2% BSA, which was inadequate, so alternative approaches were assessed. Across the three selected alternative blocking reagents, two are commonly used with monoclonal antibodies in ELISA applications. Seablock, derived from Atlantic salmon serum (*Salmo salar*) has application in ELISA, Western Blot, and IHC assays. Alternatively, a higher concentration (5%) of bovine serum albumin (BSA, mw 66kDa), which is commonly used in immunoassays, was assessed.

It was considered that blocking reagents used for mAb:target protein interactions may not be effective in preventing unwanted binding between SOMAmers and target

proteins, due to the relative sizes of the molecules. A blocking reagent may provide sufficient occlusion of the antigen surface to prevent non-specific binding by a 150 kDa IgG mAb but may be ineffective for the smaller mass and physical size of SOMAmers (~20 kDa). Therefore, a blocking reagent that is smaller in size (500 Da) and mass, such as deoxynucleotide triphosphates (dNTPs), was assessed as a third alternative. However, these three alternative blocking reagents were insufficiently effective in preventing undesired non-specific binding in general. Although there were high levels of binding between SARS-CoV-2 spike selected SOMAmers and SARS-CoV-2 spike target, non-specific binding was also present with SARS-CoV N (C11, F11) and SARS-CoV-2 N (C8) selected SOMAmers (**Figure 3.12A**). In general, the three blocking agents appeared to have little effect when compared with the absence of blocking agent (**Figure 3.12A**). The introduction of unrelated target antigens OC43 spike and IBV N also demonstrated non-specific binding by SOMAmers (**Figure 3.12B and 3.12C**). Indeed, in the case of OC43 target antigen, it appeared that some blocking agents enhanced non-specific binding, such as 5% BSA with SARS-CoV-2 spike SOMAmers and dNTPs with N SOMAmers (**Figure 3.12B**). Lower levels of binding occurred overall between SOMAmers and IBV N target protein, compared with the two other targets, with consistent levels of non-specific binding irrespective of the blocking agent used or its absence (**Figure 3.12C**).

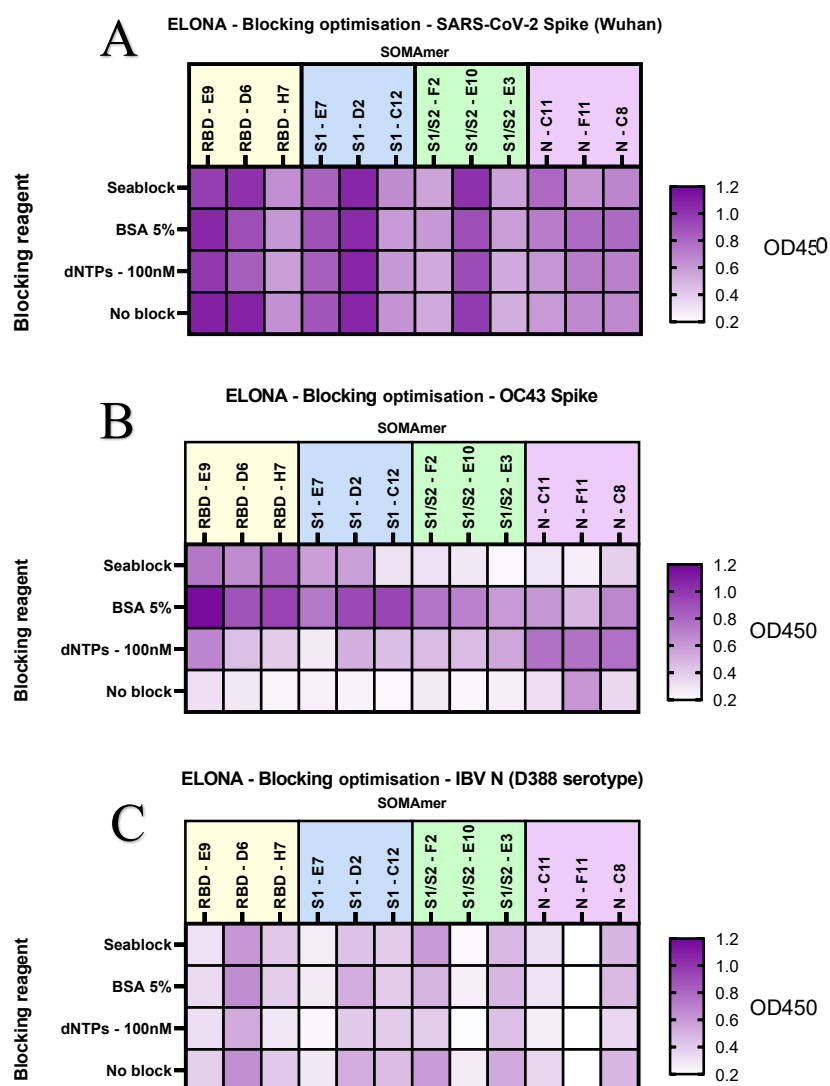


Figure 3.12: Application of alternative blocking reagents in ELONA development using SOMAmers.

The ELONA format was as described in Figure 3.6, conditions included; the viral coating antigen (80 ng/well) was SARS-CoV-2 Wuhan-Hu-1 spike (panel A), and as control antigens for non-specific binding, OC43 spike (panel B) and IBV nucleocapsid (panel C); SOMAmers selected against SARS-CoV-2 RBD, S1 and S1/S2 and nucleocapsid were applied at 50 μ L/well of 10 nM stock; the blocking agents included 50 μ L/well of 2% BSA; 50 μ L of stock concentration Seablock, 50 μ L/well of 100 nM dNTPs. Streptavidin-HRP was added at 50 μ L/well of 1/400 dilution of 1 mg/mL stock; the washing buffer used between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. Binding response data averaged from triplicate technical repeats and presented as raw OD signal. The colour intensity on the heatmap corresponds to binding signal.

Following discussion of the challenges of non-specific binding with industrial partners within the project, a proprietary blocking reagent called Z-block was provided for assessment. Z-block is a ssDNA product, developed in parallel with SOMAmers to provide a blocking reagent to prevent non-specific interactions through polyanionic inhibition. Z-Block has been used as a blocking reagent in confocal microscopy methods using ssDNA SOMAmers [114].

3.2.5 Inclusion of Z-block reduces non-specific interactions between SOMAmers and target antigens

The investigation into non-specific binding by SOMAmers in the ELONA format was continued. The effect of using Z-block was assessed and a broader range of controls were used to identify potential steps in the ELONA which were contributing to non-specific binding. The SARS-CoV-2 RBD selected SOMAmer E9 was chosen for this study as it had displayed strong binding to SARS-CoV-2 spike protein in previous experiments.

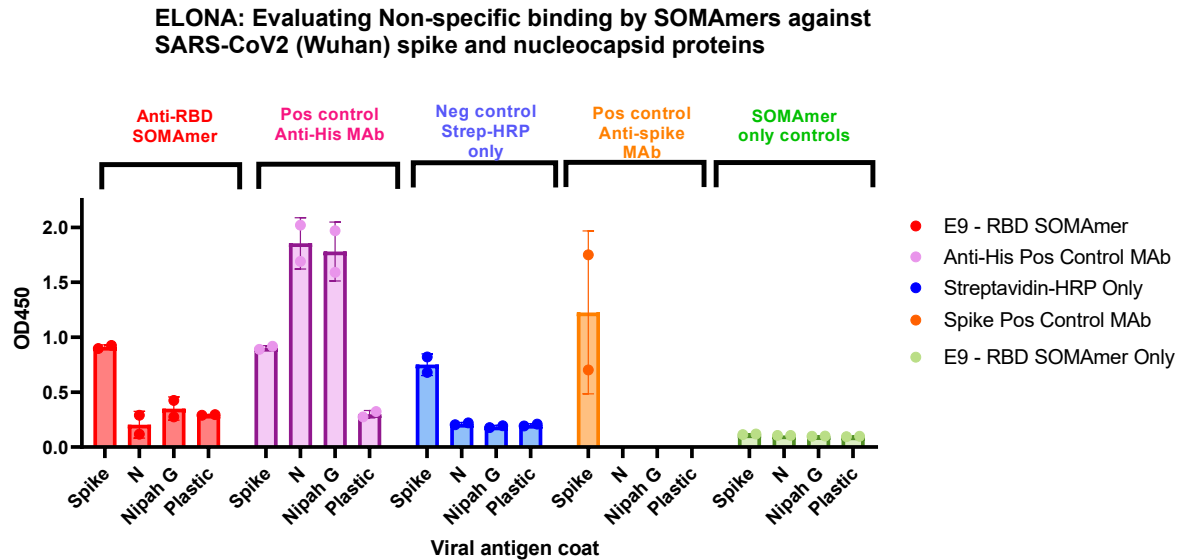


Figure 3.13: Evaluating non-specific binding by a SOMAmer E9 against SARS-CoV-2 FL spike and nucleocapsid.

The ELONA format was as described in Figure 3.6; conditions included viral coating antigen (80 ng/well) of SARS-CoV-2 spike, and as control antigens for non-specific interactions, SARS-CoV-2 nucleocapsid and Nipah virus G protein; SARS-CoV-2 spike SOMAmer (E9, red) was applied at 50 μ L/well of 10 nM stock; anti-His mAb (pink) and anti-RBD mAb (CR3022, orange) were used as positive controls. The blocking agent was 50 μ L/well of 50 μ M Z-block; streptavidin-HRP was added at 50 μ L/well of 1/400 dilution of 1 mg/mL stock or for wells using mAb, 50 μ L/well of 1/1000 dilution of stock of anti-mouse IgG-HRP was used as a secondary antibody ; the washing buffer used between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L/well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. Other controls included wells with no target protein (plastic), wells with target antigen and streptavidin: HRP, but with no SOMAmer or mAb (blue), and wells with target antigen and SOMAmer, but with no conjugate containing HRP (green), streptavidin-HRP conjugate only control with target antigens (blue). Binding response data averaged from duplicate technical repeats and presented as raw OD signal.

Following inclusion of Z-block into the ELONA assay, binding interactions between the SARS-CoV-2 spike selected SOMAmer E9 to SARS-CoV-2 spike target appeared to be retained (OD450 ~1) and non-specific binding to unrelated viral antigen targets (SARS-CoV-2 N and Nipah G) was reduced to an acceptable level of signal (**Figure 3.13**, red bars) compared with previous experiments (**Figure 3.10**). The anti-RBD SOMAmer E9 binding against SARS-CoV-2 N and an unrelated viral glycoprotein, Nipah G (**Figure 3.13**) demonstrated only low levels of non-specific binding. This

indicated SOMAmer E9 appeared to be binding with greater specificity in the presence of Z-block.

A positive control for antigen loading was used to confirm the abundance of protein immobilised onto the ELONA plate surface. Coated target antigen in wells was incubated with anti-His tag mAb followed by a species specific (anti-mouse) secondary antibody conjugated to HRP. This detection was independent of streptavidin-HRP and indicated adequate levels of binding confirming antigen presence (**Figure 3.13**, purple bars).

To examine the impact of detection reagents, a negative control was incorporated to determine if the streptavidin-HRP component was contributing to the observed non-specific binding signal. Surprisingly, this demonstrated that streptavidin-HRP (used at 1/400 dilution) displayed binding to SARS-CoV-2 full length spike (**Figure 3.13**, blue bars) when SOMAmers were not present in the reaction. In assay wells with no biotin/biotinylated molecules outside of the applied SOMAmers, this interaction between streptavidin-HRP and spike (**Figure 3.13**, blue bars) was equal in signal strength to the binding observed between spike SOMAmers to spike antigen (**Figure 3.13**, red bars). This indicated that binding signal thought to be due to specific SOMAmer-antigen interaction, could be an artifact of streptavidin-HRP binding directly to spike protein in the absence of adequate blocking. There was less evidence of this non-specific binding to the other viral antigen targets, SARS-CoV-2 N and Nipah G (**Figure 3.13**, blue bars).

The positive control of anti-SARS-CoV RBD mAb (CR3022) demonstrated binding to SARS-CoV-2 spike protein, but not nucleoprotein, Nipah G or plastic (**Figure 3.13**, orange bars). Again, the mAb CR3022 was followed by a species specific (anti-mouse) secondary antibody conjugated to HRP, so the reaction was independent of

streptavidin-HRP and therefore not affected by the observed non-specific binding seen between streptavidin-HRP and SARS-CoV-2 full length spike. As a further control, target viral antigens were also incubated with SOMAmer E9, but without streptavidin-HRP conjugate or any other source of HRP. As expected, there was minimal non-specific binding signal (**Figure 3.13**, green bars). Further investigation into the non-specific binding between SARS-CoV-2 spike protein and streptavidin-HRP was required to reduce non-specific binding in ELONA.

3.2.6 Titration of streptavidin-HRP against SARS-CoV-2 Wuhan-Hu-1 spike to evaluate cross reactive binding.

All SOMAmers within the library were conjugated to biotin, which provided the only detection method in ELONA, i.e. via streptavidin-HRP and TMB substrate detection. However, non-specific binding between SARS-CoV-2 spike protein and streptavidin-HRP was problematic, by potentially masking the signal from any specific SOMAmer-antigen interaction. Reducing streptavidin-HRP concentrations in the ELONA and using different sources of streptavidin-HRP were investigated in an attempt to reduce non-specific binding.

Alternate stocks (S911 and Ultra) and concentrations of streptavidin-HRP were assessed over a range of dilutions from 1/400 to 1/51200 of stock. ELONA reactions with streptavidin-HRP in the absence of SOMAmer indicated reducing levels of non-specific binding to SARS-CoV-2 spike target antigen as the conjugate was diluted (**Figure 3.14**, blue and purple bars). Streptavidin-HRP concentrations of 1/1600 and 1/3200 stock (**Figure 3.14**, red boxes) were chosen for further experiments as non-specific binding to SARS-CoV-2 spike protein was reduced at these dilutions.

However, the ELONA assays including SOMAmer E9 produced a comparable signal with assays without SOMAmer (**Figure 3.14**, blue and purple bars compared with pink bars). This indicated that specific binding between SOMAmer E9 and SARS-CoV-2

spike target antigen was either not occurring or was not visible. It was considered possible that the reduction in streptavidin-HRP concentration from 1/400 to 1/1600 or 1/3200 may require a compensatory increase in other reaction components, such as target antigen or SOMAmer concentration, to obtain a specific binding signal.

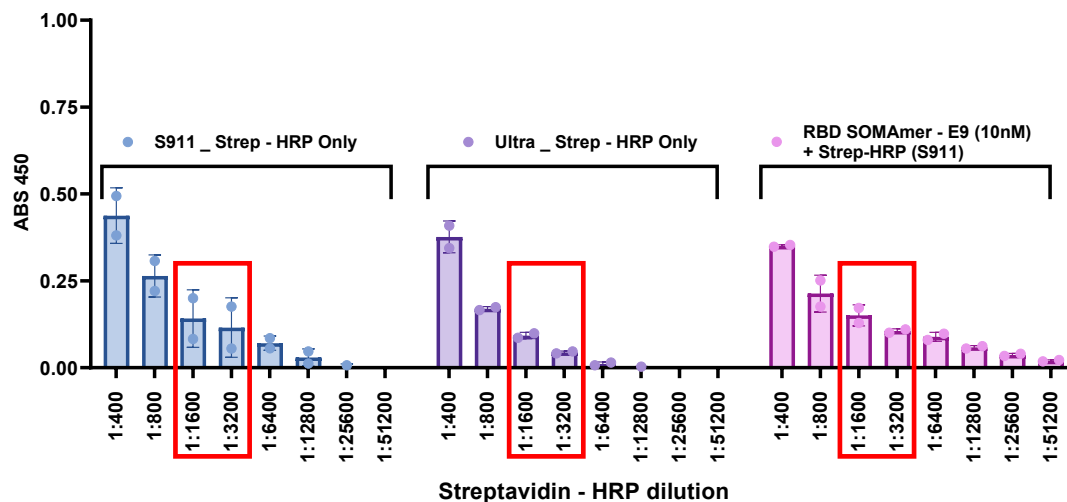


Figure 3.14: Evaluating binding of reducing concentrations of streptavidin-HRP conjugate to SARS-CoV-2 spike in ELONA format.

The ELONA format was as described in Figure 3.6; conditions included viral coating antigen (80 ng/well) of SARS-CoV-2 Wuhan-Hu-1 spike in all wells, SARS-CoV-2 RBD SOMAmer (E9) was applied at 50 μ L/well of 10 nM stock (pink bars); the blocking agent was 50 μ L/well of 50 μ M Z-block; streptavidin-HRP was added at 50 μ L/well of 1/400 to 1/51200 dilutions of 1 mg/mL stock; the washing buffer between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution read at absorbance 450 nm. Binding response data averaged from duplicate technical repeats and presented as raw OD signal.

The next step in ELONA assay development aimed to retain the reduced non-specific binding resulting from reduced concentrations of streptavidin-HRP and to investigate if increased concentrations of SOMAmer could lead to a visible specific binding signal. A range of concentrations of SOMAmer E9 from 40 nM to 0.75 nM was investigated in ELONA experiments using 1/1600 and 1/3200 dilutions of stock S911 streptavidin-HRP, and a 1/1600 dilution of a new stock of Ultra streptavidin-HRP.

As expected, a 1/1600 dilution of streptavidin-HRP conjugate displayed stronger binding signal responses than a 1/3200 dilution and there was a reduction in binding signal as SOMAmer concentration reduced (**Figure 3.15**, blue and purple bars). The new stock of Ultra streptavidin-HRP conjugate at a dilution of 1/1600 produced a higher signal overall, which reduced as SOMAmer E9 concentration was reduced (**Figure 3.15**, pink bars) but still showed a low level of binding in the absence of SOMAmer E9. The marked difference in response between streptavidin-HRP sources suggest that the older stock may have been sub-optimal in performance.

The encouraging SOMAmer concentration-dependent binding response at a single streptavidin-HRP concentration (**Figure 3.15**, pink bars) indicated that binding was not solely due to non-specific streptavidin-HRP interactions with SARS-CoV-2 spike target antigen, although a level of residual binding remained in the absence of SOMAmers. This was dealt with by subtracting non-specific binding seen in the 'no SOMAmer control' (**Figure 3.15**, pink bar on right hand side) from all SOMAmer containing binding absorbances (**Figure 3.15**, other pink bars) resulting in the 'secondary background subtracted' absorbances (**Figure 3.15**, black bars). The concentration of SOMAmers used in subsequent studies was determined as 10 nM, which balanced efficient use of precious reagents with production of an adequate signal (~1 OD at 450 nm).

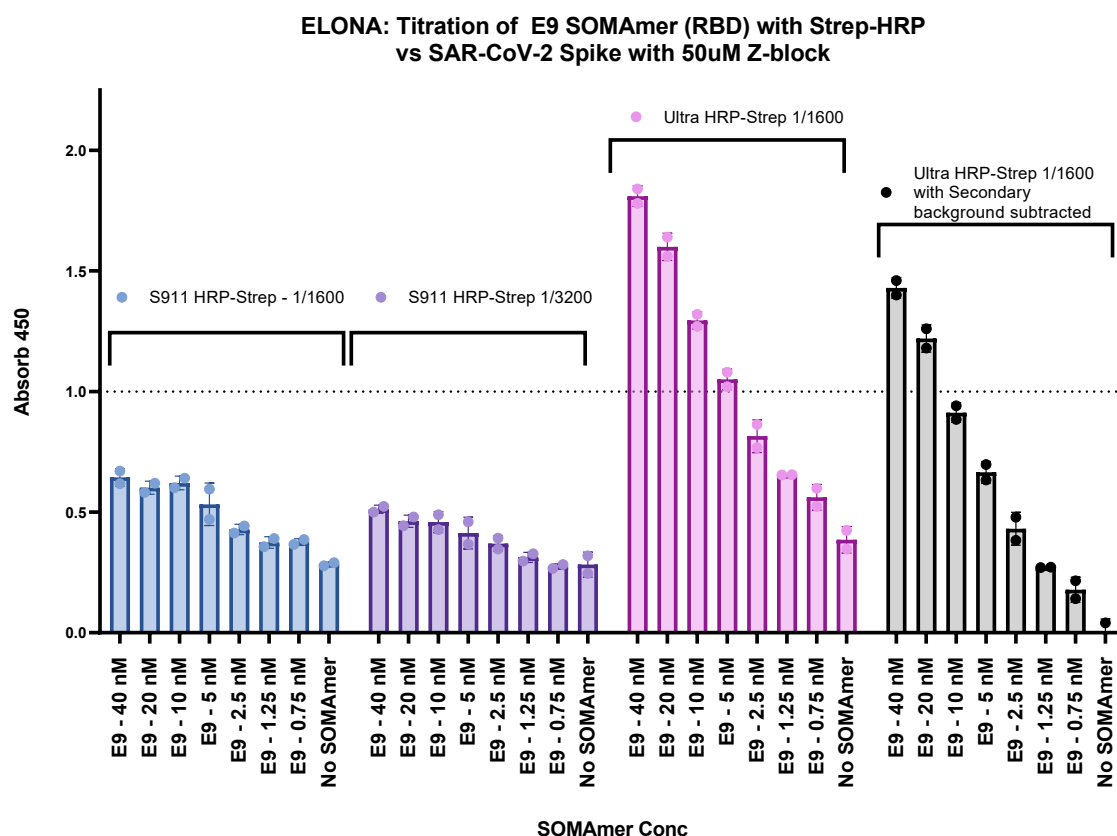


Figure 3.15: Titration of SOMAmer E9 concentration to determine impact on signal strength.

The ELONA format was as described in Figure 3.6; conditions included viral coating antigen (80 ng/well) SARS-CoV-2 Wuhan-Hu-1 spike in all wells; anti-SARS-CoV-2 RBD SOMAmer (E9) applied at 50 μ L/well in a range of concentrations of 40 nM to 0.75 nM. The blocking agent was 50 μ L/well of 50 μ M Z-block; streptavidin-HRP was added at 50 μ L/well of 1/1600 or 1/3200 dilution of 1 mg/mL stock; the washing buffer used between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. Binding response data averaged from duplicate technical repeats and presented as raw OD signal.

The “no SOMAmer” control absorbance value (pink bar on right hand side) was subtracted from all other assay wells (pink bars) to provide a subtracted signal for each SOMAmer concentration, presented as raw OD450 values (black bars).

3.2.7 Investigation of optimal concentration of Z-block in ELONA

Following adjustment of streptavidin-HRP concentration and SOMAmer quantity in the ELONA, the concentration of Z-block used as blocking agent was further investigated. Two ranges of dilutions of Z-block as blocking agent in ELONA were examined where SARS-CoV-2 RBD SOMAmer E9 was applied to SARS-CoV-2 RBD, spike and nucleoprotein viral protein coated assay wells.

Both ranges of Z-block as blocking agent (50 μ M to 0.1 μ M, and 50 μ M to 0.781 μ M) ensured retention of SOMAmer E9 binding to SARS-CoV-2 RBD and spike target antigens (**Figure 3.16 A and B**). Non-specific binding of SOMAmer E9 to SARS-CoV-2 nucleoprotein was prevented at higher concentrations of Z-block (**Figure 3.16 A and B**), with the concentration of 10 μ M chosen for future experiments. In the absence of Z-block, non-specific binding of SOMAmer E9 to SARS-CoV-2 nucleoprotein was evident (**Figure 3.16 A and B**), reinforcing the requirement for this blocking reagent in achieving assay specificity.

The control wells using anti-His tag mAb and anti-SARS-CoV RBD mAb indicated adequate levels of coating antigen and specifically the presence of RBD/spike protein respectively.

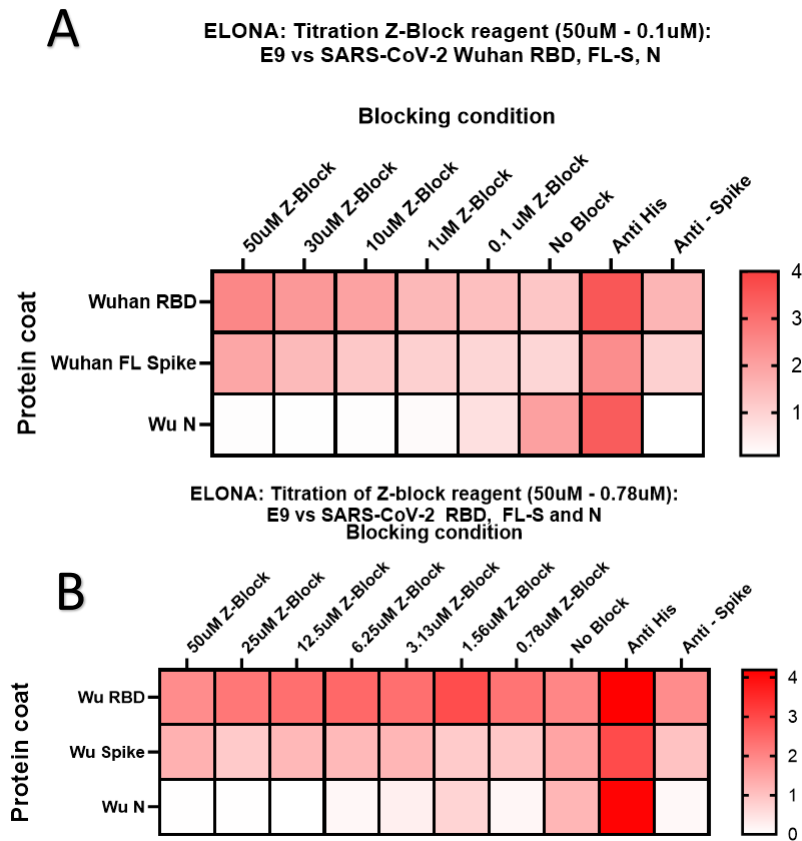


Figure 3.16: Titration of Z-block blocking reagent using SOMAmer E9 and single concentrations of Wuhan-Hu-1 RBD, spike and nucleocapsid

The ELONA format was as described in Figure 3.6; conditions included viral coating antigens (80 ng/well) SARS-CoV-2 Wuhan-Hu-1 RBD, spike, and nucleocapsid.; anti-SARS-CoV-2 RBD SOMAmer (E9) was applied at 50 μ L/well of 10 nM stock; anti-His mAb and anti-SARS-CoV RBD mAb (CR3022) were used as positive controls. The blocking agent was 50 μ L/well of a dilution range of 50 μ M to 0.1 μ M Z-block (panel A) and 50 μ M to 0.078 μ M Z-block (panel B). Streptavidin-HRP was added at 50 μ L/well of 1/1600 dilution of 1 mg/mL stock, or for wells using mAb, 50 μ L/well of 1/1000 stock of anti-mouse IgG-HRP was used as a secondary antibody; the washing buffer used between each step was 0.05% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. Binding response data averaged from triplicate technical repeats and presented as raw OD signal.

The colour intensity on the heatmap corresponds to binding signal, with streptavidin-HRP only non-specific binding value subtracted.

3.2.8 Z-block and enhanced washing prevent non-specific binding

At this stage of ELONA development, there was still non-specific binding evident between SARS-CoV-2 spike protein and streptavidin-HRP, although this had been reduced by use of the blocking agent, Z-block. An additional approach to reducing non-specific binding was to increase the detergent concentration in the washing buffer from 0.05% Tween-20 to 0.5% Tween-20.

A wider range of SOMAmers were used for this study, incorporating SOMAmers selected against RBD (E9, D6), S1(E7, D3) and S1/S2 (F2, E12) as well as a control SOMAmer selected against SARS-CoV nucleocapsid (F11). Wells were coated with SARS-CoV-2 RBD, spike, or nucleocapsid and the unrelated antigen, Nipah G.

As expected, SOMAmers selected against RBD (E9 and D6) showed binding to RBD, and SOMAmers selected against S1(E7 and D3) or S1/S2 (F2 and E12) showed binding to spike protein (**Figure 3.17**). The two S1-selected SOMAmers (E7 and D3) also bound to RBD, a region within the spike protein. These RBD:S1:S1/S2-selected SOMAmers showed no or minimal non-specific binding to SARS-CoV-2 N or Nipah G (**Figure 3.17**).

Reflecting the high sequence homology between SARS-CoV N and SARS-CoV-2 N, the SARS-CoV N-selected SOMAmer (F11) bound to SARS-CoV-2 N. However, there was no non-specific binding by the F11 SOMAmer to RBD, spike or Nipah G (**Figure 3.17**).

The anti-His mAb positive control indicated adequate coating protein loading, and the anti-RBD mAb CR3022 positive control showed binding to RBD and spike coating antigens (**Figure 3.17**).

However, there remained some non-specific binding between streptavidin:HRP and SARS-CoV-2 spike protein, although this was not evident with RBD, N or Nipah G (**Figure 3.17**).

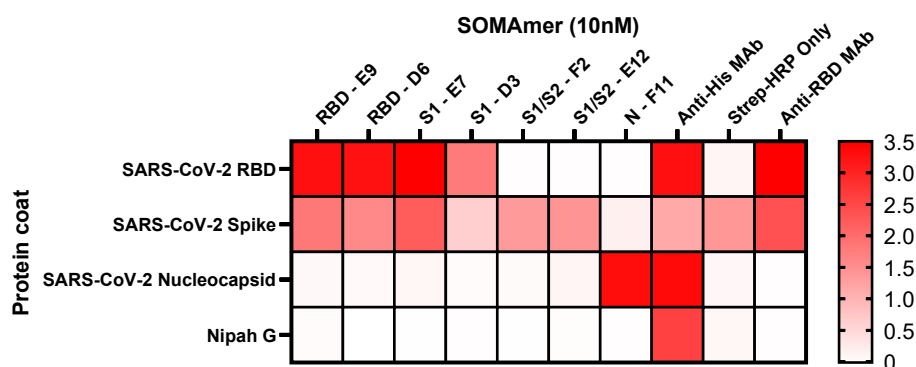


Figure 3.17: ELONA with enhanced concentration of detergent in wash buffer for E9, D6, E7, D3, F2, E12, C8 SOMAmer binding

ELONA format as Figure 3.6, conditions included; viral coating antigen (80 nG per well) SARS-CoV-2 Wuhan-Hu-1 RBD, spike, and nucleocapsid, Nipah G; anti-SARS-CoV-2 SOMAMers for RBD (E9, D6), S1 (E7,D3), S1/S2 (F2, E12), and N (F11) applied at 50 μ L/well of 10 nM stock; anti-His mAb and anti-SARS-CoV RBD mAb (CR3022) were used as positive controls. The blocking agent was 50 μ L of 10 μ M Z-Block; streptavidin-HRP was added at 50 μ L/well of 1 in 1/1600 dilution of 1 mg/mL stock, or where mAbs were used, 50 μ L/well of 1/1000 stock of anti-mouse IgG-HRP was used as a secondary antibody; washing buffer between each step was 0.5% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. The colour intensity on the heatmap corresponds to binding signal. Streptavidin-HRP background binding was subtracted from assay wells with SOMAmer present, for each viral coating protein. Binding response data averaged from triplicate technical repeats and presented as raw OD signal.

As a last stage in ELONA development, the role of Z-block in reducing non-specific binding was re-examined in the presence of the enhanced washing procedure, where the Tween-20 concentration had been increased from 0.05% to 0.5%. The SARS-CoV-2 RBD SOMAmer E9 was used in this study and compared with wells without SOMAmer. The coating antigens were SARS-CoV-2 spike or N as a negative control, and blocking reagents Z-block, 2% BSA or PBS as a control were compared.

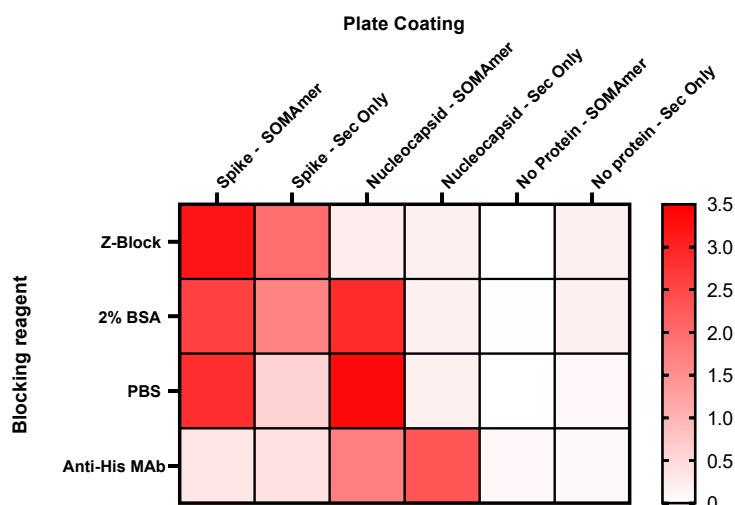
As seen in previous experiments, SOMAmer E9 apparently bound spike protein with Z-block, 2% BSA or PBS, but in the absence of SOMAmer, non-specific binding occurred between spike protein and streptavidin-HRP (**Figure 3.18A**). However, in the presence of Z-block, non-specific binding of SOMAmer E9 to SARS-CoV-2 N was reduced compared with 2% BSA or PBS as blocking agents (**Figure 3.18A**). This re-

confirmed the importance of Z-block as an effective blocking agent to reduce non-specific binding in the developed ELONA enabling specific binding to be evident.

There was no evidence of binding reactions to SARS-CoV-2 N by streptavidin-HRP in the absence of SOMAmer, or any non-specific binding to well surfaces in the absence of coating protein (**Figure 3.18A**).

As above, to address the residual non-specific binding between spike protein and streptavidin-HRP, the absorbance reading from this reaction was subtracted from reactions containing these components (**Figure 3.18B**) enabling specific binding to be visualised.

A Blocking reagent evaluation in ELONA:E9 SOMAmer binding SARS-CoV-2 Spike and N with 0.5% tween-20 wash (HRP-Strep background not subtracted)



B Blocking reagent evaluation in ELONA:E9 SOMAmer binding SARS-CoV-2 Spike and N with 0.5% tween-20 washing. (HRP-Strep background subtracted)

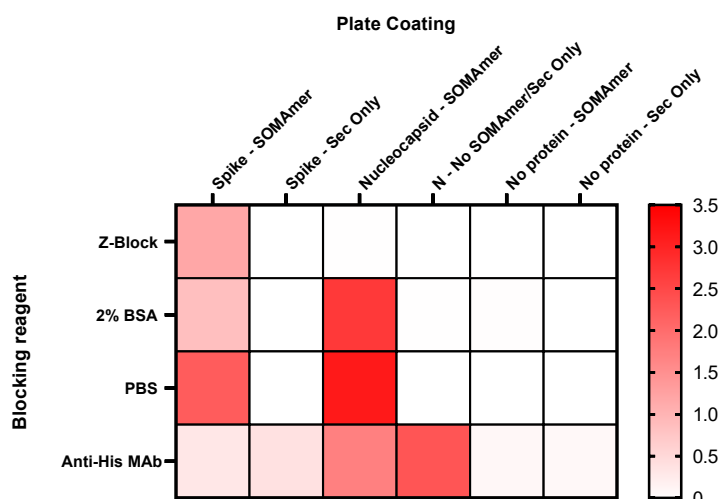


Figure 3.18: ELONA with enhanced washing and differing blocking reagents

The ELONA format was as described in Figure 3.6; conditions included viral coating antigen (80 ng/well) of SARS-CoV-2 Wuhan-Hu-1 spike and nucleocapsid; anti SARS-CoV-2 RBD SOMAmer (E9) was applied at 50 μ L/well from a 10 nM stock; anti-His mAb was used as positive control. Wells were blocked with 50 μ L/well of 10 μ M Z-Block stock, 2% BSA, or PBS. Streptavidin-HRP was added at 50 μ L/well of 1 in 1/1600 dilution of 1 mg/mL stock, or where mAbs were used, 50 μ L/well of 1/1000 stock of anti-mouse IgG-HRP was used as a secondary antibody; washing buffer between each step was 0.5% Tween-20 in PBS. Following incubation with 100 μ L per well of Ultra TMB for 5 minutes of development, and neutralisation with 1 M sulphuric acid solution, absorbance was read at 450 nm. The colour intensity on the heatmap corresponds to binding signal. Binding response data averaged from triplicate technical repeats and presented as raw OD signal.

Figure 3.18B presents the same data as Figure 3.18A, with streptavidin-HRP:spike non-specific binding subtracted from assay wells with SOMAmer present.

3.2.9 Summary diagrams of final ELONA arrangement for use in subsequent screening studies

Initially multiple interactions contributed to non-specific binding within the ELONA development (**Figure 3.19A**). These included binding of SOMAmers to unrelated viral antigens and non-specific binding between SARS-CoV-2 spike protein and the reagent streptavidin-HRP.

Where SARS-CoV-2 spike and nucleocapsid SOMAmers were observed as binding to unrelated viral antigens, this was reduced by the addition of a ssDNA polyanionic competitor called Z-block which reduced unwanted interactions (**Figure 3.19A**). Secondly, the challenge during ELONA development of streptavidin-HRPs affinity for full length SARS-CoV2 spike protein contributing to non-specific binding (**Figure 3.12**) was addressed by increasing concentrations of detergent within the washing buffer. The final format of the developed ELONA used for further SOMAmer screening is shown in **Figure 3.19B**.

ELONA Blocking and washing optimisation

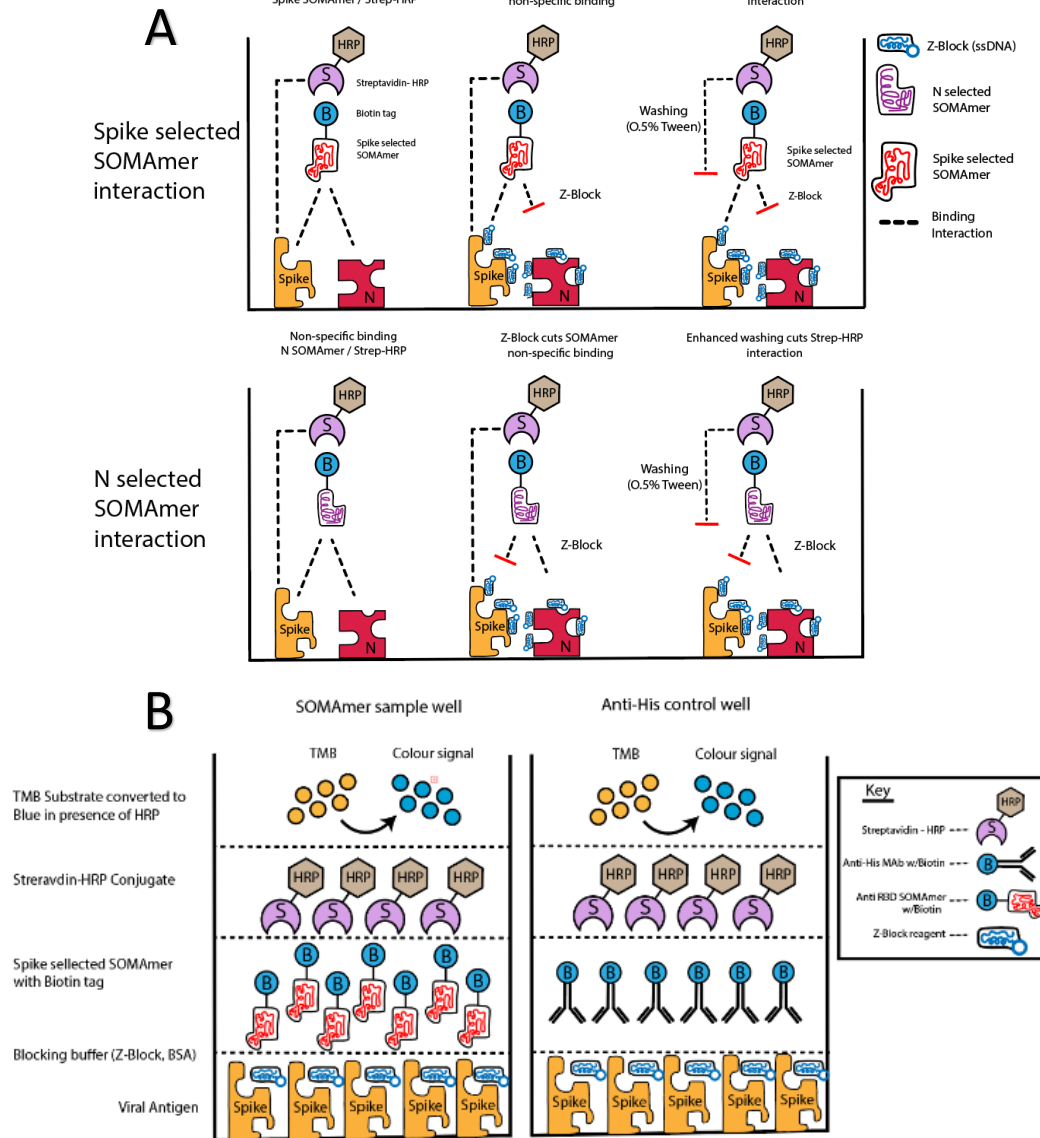


Figure 3.19: Illustration of ELONA non-specific interactions resolution

A. Demonstration of ELONA interactions resulting in non-specific binding for both spike selected SOMAmers and for nucleocapsid SOMAmers shown to be prevented by addition of Z-block buffer and a higher concentration of Tween-20 in the washing buffer. Within figure 19A, unwanted interactions demonstrated with dashed lines are reduced (red bar) resulting in specific interactions by SOMAmers.

B. Summary diagram of the final ELONA assay developed for the evaluation of SARS-CoV-2 Wuhan-Hu-1 SOMAmers on divergent coronaviruses.

3.3 - Discussion

Assay development for the project was dependent on a reliable source of purified viral antigens from coronaviruses and an unrelated virus as a control (**Table 3.1**). This was achieved by a combination of approaches, ranging from the full process of cloning, expression and purification through to generous gifts from colleagues.

A significant challenge in the development of ELONA is unwanted cross-reactivity (non-specific binding), which was encountered here during initial SOMAmer binding studies via ELONA (Enzyme-Linked Oligonucleotide Assay) (**Figures 3.9, 3.10, 3.11**). As DNA-based affinity reagents, SOMAmer binding is driven by three-dimensional conformation and hydrophobic chemical modifications for specific epitope recognition. These properties are reported to drive specific recognition of epitopes, with sensitivity to subtle changes in protein conformation. Determining if SOMAmers can demonstrate specific binding requires distinguishing between cross-reactive binding between proteins that share related epitopes and non-specific binding, the latter being a situation where a SOMAmer displays binding to unrelated proteins compared to their SELEX target.

As ssDNA, all SOMAmers can be described as polyanionic, with an overall negative electrical charge, thereby interacting strongly with positively charged patches on protein surfaces. This non-specific binding can be mitigated using blocking agents that occupy these charged regions without interfering with specific SELEX-derived binding sites.

In ELISA systems utilising monoclonal antibodies, bovine serum albumin (BSA) is commonly used as a blocking agent due to its small size (66 kDa), stability, and mildly anionic nature. As a protein, albumin provides a steric and electrostatic barrier, preventing non-specific interactions and ensuring that only true antigen–antibody

interactions produce a measurable signal. In contrast, for ELONA and lateral flow assay formats, literature [115] and experimental data suggested that BSA alone is not sufficient to prevent SOMAmer non-specific interactions. Therefore, optimal blocking conditions had to be empirically determined.

Within the SOMAscan platform, the polyanionic competitor (Z-block) is additionally used to eliminate non-specific interactions through a “kinetic challenge” process. During this stage, weakly bound SOMAmers are displaced by the competitor, also referred to as a “polyanionic competitor”, whereas high-affinity, epitope-specific SOMAmers remain bound. The application of this polyanionic competitor reagent, referred to as Z-block, is incorporated within the SOMAscan assay which through its high multiplexing capacity can detect up to 11,000 distinct biomarkers [116].

Experimental findings identified two sources of non-specific binding within the ELONA assay developed here, which were successfully mitigated through the optimisation of streptavidin-HRP concentration (**Figure 3.14**), enhanced washing buffer and the ssDNA-specific blocking reagent (Z-block) (**Figures 3.16, 3.17, 3.18**). These refinements ensured that the binding observed was specific to the intended viral protein targets. Overcoming these non-specific interactions was essential for the success of the project; without this, investigation of the binding properties of the SOMAmer library and progression toward a prototype diagnostic would not have been possible. The absence of binding to unrelated coronavirus or non-coronavirus proteins demonstrated the binding discrimination and specificity of the SOMAmers.

During ELONA development, ELISA literature with similar immunostaining workflows was used to guide the selection of HRP as the detection enzyme and TMB as the colourimetric substrate [111]. As the provided library of SOMAmers were all biotin conjugated, the detection method used was streptavidin conjugated to horseradish

peroxidase (HRP). During development, it became clear that at high concentrations this conjugate could interact with the antigen used for SOMAmer library SELEX, specifically the spike glycoprotein (**Figure 3.13**).

Non-specific binding between target antigens and detection enzyme conjugates is uncommon in assay development according to literature sources, although it should be controlled for as an important step in assay development. Titration to optimise and reduce the streptavidin–HRP reagent concentration, along with more stringent washing buffers, proved to be effective mitigation strategies, reducing weakly bound background material. Ultimately, for full-length spike, not all non-specific binding could be removed; however, the developed ELONA conditions allowed the background signal to be lower than the SOMAmer signal, allowing background subtraction without masking assay performance. No such challenge was experienced during the use of SARS-CoV-2 RBD as compared with FL-spike in ELONA.

Within this assay, we demonstrated the development of a specific and sensitive ELONA-based assay for effectively screening candidate SOMAmers. The purpose of this chapter, which was to develop an assay enabling the assessment of each candidate as a recognition element, was therefore achieved. This firstly confirmed aptamer function and secondly verified binding specificity, informing which SOMAmers were to be selected for further evaluation. The developed ELONA assay (**Figure 3.19**) provided a scalable approach, assessing and validating SOMAmer performance, providing insight into optimal buffer conditions and allowing future identification of the strongest SOMAmer candidates for transition into a rapid diagnostic lateral flow context.

Chapter 4: Binding evaluation of SOMAmers against divergent coronaviruses

4.1 Introduction

The respiratory coronavirus SARS-CoV-2 represents one of the most consequential pathogens in medical history, characterised by a high basic reproduction number (R_0), a broad spectrum of clinical manifestations, and substantial mutational capacity, all of which have collectively enabled it to evade and outpace successive public health interventions. A critical component of pandemic preparedness is the development of adaptable technologies such as diagnostics, vaccines, and therapeutics which enable rapid responses to pre-pandemic, pandemic, and post-endemic threats.

In pandemic control, low-cost lateral flow-based diagnostics address two central challenges: accessibility during periods of high global demand and scalability when millions of tests are required for effective intervention. Current mass diagnostics rely on monoclonal antibodies (mAbs), which provide high affinity and specificity but are inherently slower to develop, validate, and manufacture for integration into diagnostic platforms, rendering them susceptible to potential immune escape driven by viral evolution.

Aptamers, in contrast, can be developed more rapidly and offer an adaptable, lower-cost alternative. Often termed “chemical antibodies”, aptamers are nucleic acid molecules that fold into specific three-dimensional structures, enabling them to bind a wide range of targets, including proteins, with high affinity [117].

Their isolation relies on Systematic Evolution of Ligands by Exponential Enrichment (SELEX), an in vitro method that can be adapted for the rapid selection of aptamers against new targets, including emerging viral variants. Aptamer selection induces and

drives single-stranded DNA (ssDNA) folding to become specific to the protein target presented. This capacity underpins the ability of aptamers to be rapidly redeveloped, allowing new SELEX rounds to be performed to adapt to newly mutated protein targets. In the context of this research project, however, no such regeneration was performed; a library of 45 SOMAmers selected against the SARS-CoV-2 Wuhan-Hu-1 (19A) variant spike protein was provided for study.

Notably, certain broadly neutralising antibodies and aptamers have been shown to exhibit cross-reactive binding across diverse species within viral families [96], indicating epitope conservation. Demonstrating such cross-reactivity would position SOMAmers (a class of modified aptamers) as a powerful and durable platform for future pandemic countermeasures.

Viral evolution was closely studied during the SARS-CoV-2 pandemic and produced a clear phylogenetic history. This information provided a model allowing evaluation of the continued performance of potential diagnostic detection recognition elements as viral mutations accumulated, an important factor during a pandemic response. Binding performance of SARS-CoV-2 Wuhan-Hu-1 SOMAmers was evaluated against SARS-CoV-2 variants and related viral species, to inform potential coronavirus pan-genus or pan-variant diagnostics.

In this chapter, I examine the capacity of SOMAmers to replace monoclonal antibodies in molecular recognition roles, to bind to a divergent subset of coronaviruses, and to demonstrate potential application towards the final objective of this thesis: the development of a SOMAmer-based prototype lateral flow test.

4.1.1 Coronavirus diversity

Screening of endemic host populations and analyses of viral evolutionary history indicate that the natural reservoirs of sarbecoviruses (betacoronaviruses) are predominantly bat species, members of the order Chiroptera. The extensive diversity of coronaviruses circulating in such populations suggests that many hundreds of as-yet unsampled viral lineages utilise these species and other wildlife as reservoirs. Such reservoirs have demonstrated a capacity for zoonotic spillover, posing risks of future transmission events to humans or other intermediate hosts, underscoring the importance of continued surveillance and characterisation of bat-associated viral diversity.

Coronaviruses are subject to mutation and reshuffling of diverse genomic segments leading to diversity that facilitates adaptation and cross-species transmission [118]. The recent COVID-19 pandemic affected millions of people worldwide and resulted in many variations and clades of SARS-CoV-2 [119].

SARS-CoV-2 variants have been arranged into a phylogenetic tree [120] (**Figure 4.1**) and representative variants (**Table 4.1**) were chosen for SOMAmer binding studies in this report, using recombinant RBD or full-length spike sequences.

In this screening study, SOMAmers were investigated to determine the breadth of binding to a divergent range of coronaviruses, including those from alpha, beta and gammacoronavirus genera. This would inform the utility of SOMAmers as molecular recognition elements in diagnostics for either a broader or narrower range of coronaviruses.

4.2 Results

4.2.1 SOMAmers bind specifically to Wuhan-Hu-1 RBD and full length spike in ELONA assay.

A library of 45 SOMAmers selected against the Wuhan-Hu-1 (19A) variant spike protein was provided for these studies. Information concerning the binding domains (RBD, S1, or S1/S2) was available for each SOMAmer, but ssDNA sequence data and epitope mapping data was not available. Nine SOMAmers in the library were generated via SELEX against the SARS-CoV-2 Wuhan-Hu-1 RBD, five against the S1 domain, and thirty-one against the S1/S2 full spike ectodomain.

To confirm information provided about the library and assess the binding of all forty-five provided SOMAmers, screening using a developed enzyme-linked oligonucleotide assay (ELONA) was performed against either the full-length ectodomain (FL) spike protein of SARS-CoV-2 (Wuhan-Hu-1) or its receptor-binding domain (**Figure 4.2**). Specific SOMAmer binding within the assay was verified using two non-specific control target antigens: SARS-CoV-2 nucleocapsid protein (N) and Nipah virus surface glycoprotein G (Bangladesh strain).

During ELONA development (**Chapter 3**) non-specific binding was observed between SARS-CoV-2 spike protein and the detection conjugate streptavidin-HRP, which was minimised as far as possible with blocking agents and washing protocols. However, low levels of residual non-specific binding remained, therefore the reaction of SARS-CoV-2 spike protein and streptavidin-HRP, in the absence of SOMAmer, was included in ELONA as a control, and OD450 absorbance value subtracted from assay well results where SOMAmers were present. Following subtraction of this non-specific binding absorbance value, resulting OD450 was expressed as a proportion of a positive control (anti-His mAb), and reported as the signal-to-positive ratio (S/P) across all ELONA plates. This method of data processing, which is used in some commercial

ELISAs [121], allows comparability across multiple coronavirus variant-, species-, and control-antigen experiments. This normalisation is important as plate-to-plate variation in antigen dilution can change the antigen abundance on the well surface, which in turn influences the binding signal responses for SOMAmer candidates.

To support SOMAmer candidate selection for downstream diagnostic applications, a binding threshold was defined using a signal-to-positive (S/P) ratio of 0.2, based on criteria commonly applied in coronavirus ELISA assays. SOMAmer candidates with $S/P > 0.2$ were considered positive for target antigen binding (**Figure 4.2A**).

The full SOMAmer library was examined for binding in ELONA against a range of coating antigens, Wuhan-Hu-1 RBD and Wuhan-Hu-1 spike, alongside the two negative controls, Wuhan-Hu-1 N and Nipah G proteins. Interestingly, of the five SOMAmers selected against the S1 domain (E7, D3, D2, D1, C12), three (E7, D3, D2) were shown to bind the Wuhan-Hu-1 RBD, while two (D1, C12) bound in non-RBD regions. Of the thirty SOMAmers selected against S1/S2, four (E11, H10, H8, H9) displayed specific binding to the RBD (**Figure 4.2A**).

In total, 16 SOMAmers (E9, D6, H7, E8, D5, H6, D4, D7, H5, E7, D3, D2, E11, H10, H8, H9) bound to the Wuhan-Hu-1 RBD and 10 SOMAmers (F2, E12, F1, D12, E10, H3, C10, H2, G10, H4) bound outside the RBD within the wider spike protein (**Figure 4.2B**). As expected, SOMAmers derived from RBD SELEX (E9, D6, H7, E8, D5, H6, D7, D4, H5) bound RBD.

SOMAmers which showed low levels of binding to target antigens, falling below the 0.2 S/P assigned cut off value (D4, C12, E5, E3, E6, E4, E2, E1, D9, H12, H1, D11, D10, D8, H11, G12, G11, G9, C9) were removed from subsequent studies.

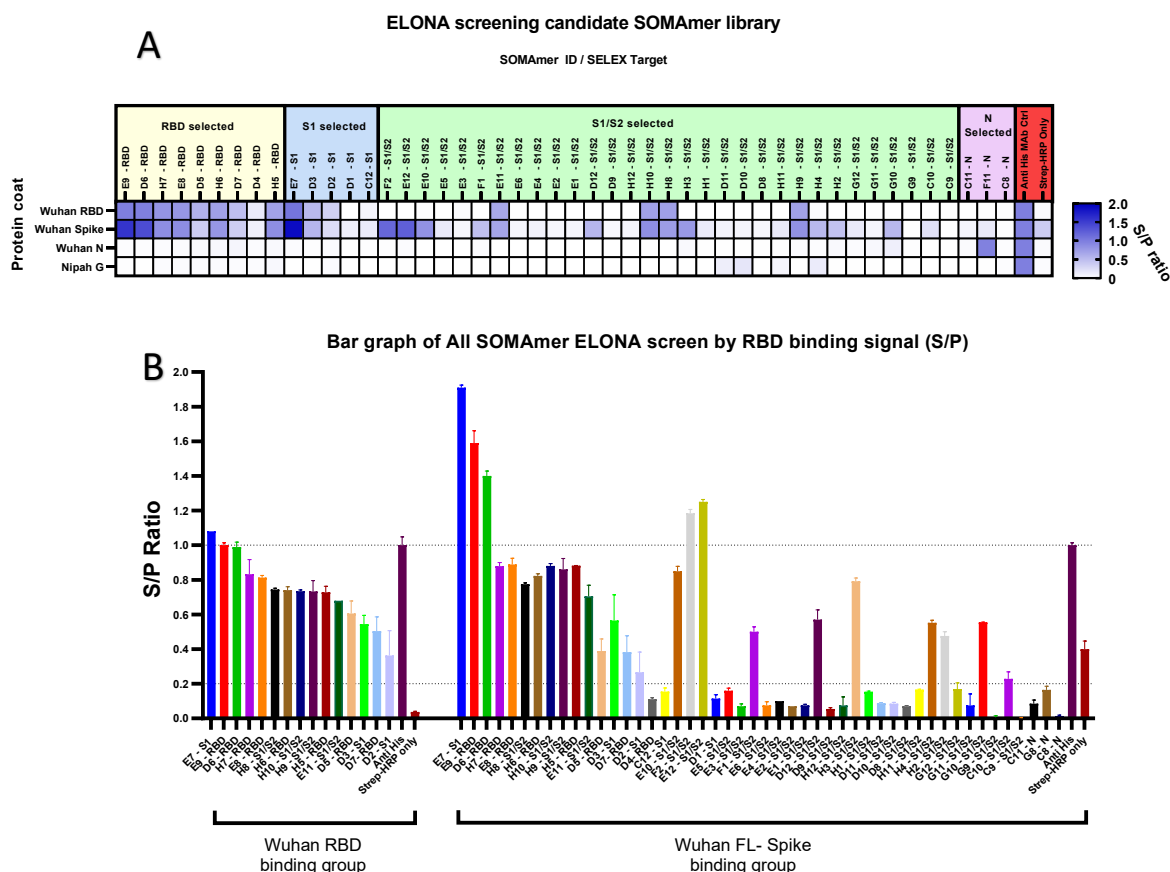


Figure 4.2: Classification of candidate SOMAmer binding by ELONA into spike epitope domains.

2A. Using ELONA, SOMAmers (80 nG/well) generated against domains of the SARS-CoV-2 spike protein were evaluated for binding to SARS-CoV-2 Wuhan-Hu-1 RBD, spike or N protein, with Nipah G as a negative control. As further controls, SOMAmers generated against SARS-CoV/SARS-CoV-2 N (C11, F11, C8), anti-His tag mAb and streptavidin-HRP were evaluated for binding to all four coating antigens. Residual streptavidin-HRP non-specific binding was subtracted from SOMAmer well raw OD450 values and expressed as the signal/positive ratio (S/P) relative to an anti-His mAb control.

A heat map of the S/P ratio showed high binding responses for some candidates, with several exceeding the positive control. As a result, the scale within **Figure 4.2A** is expressed using colour intensity corresponding to a binding abundance maximum of 2.0. The classification threshold for positive versus negative binding was set at 0.2 S/P.

2B. Bar chart representation of data from 2A, showing SOMAmers which bound to SARS-CoV-2 Wuhan-Hu-1 RBD (left side panel), and all SOMAmers binding response to SARS-CoV-2 Wuhan-Hu-1 full length spike protein (right side panel). Binding response data averaged from triplicate technical repeats. A cut off value of S/P ratio of 0.2 was set to identify SOMAmers with low binding performance.

4.2.2 Summary classification of SOMAmer candidates into binding domain groups; RBD or Non RBD binders

Down-selected RBD- or FL-spike-binding SOMAmers, derived from the summary heatmap data shown in **Figure 4.2A and B**, are displayed in **Figures 4.3A and 4.3B**. SOMAmers binding to RBD are shown in **Figure 4.3A**, and those binding outside the RBD are shown in **Figure 4.3B**. SOMAmers falling below the cutoff as low/non-reactive binders (<0.2 S/P) to screened antigens were removed from further evaluation.

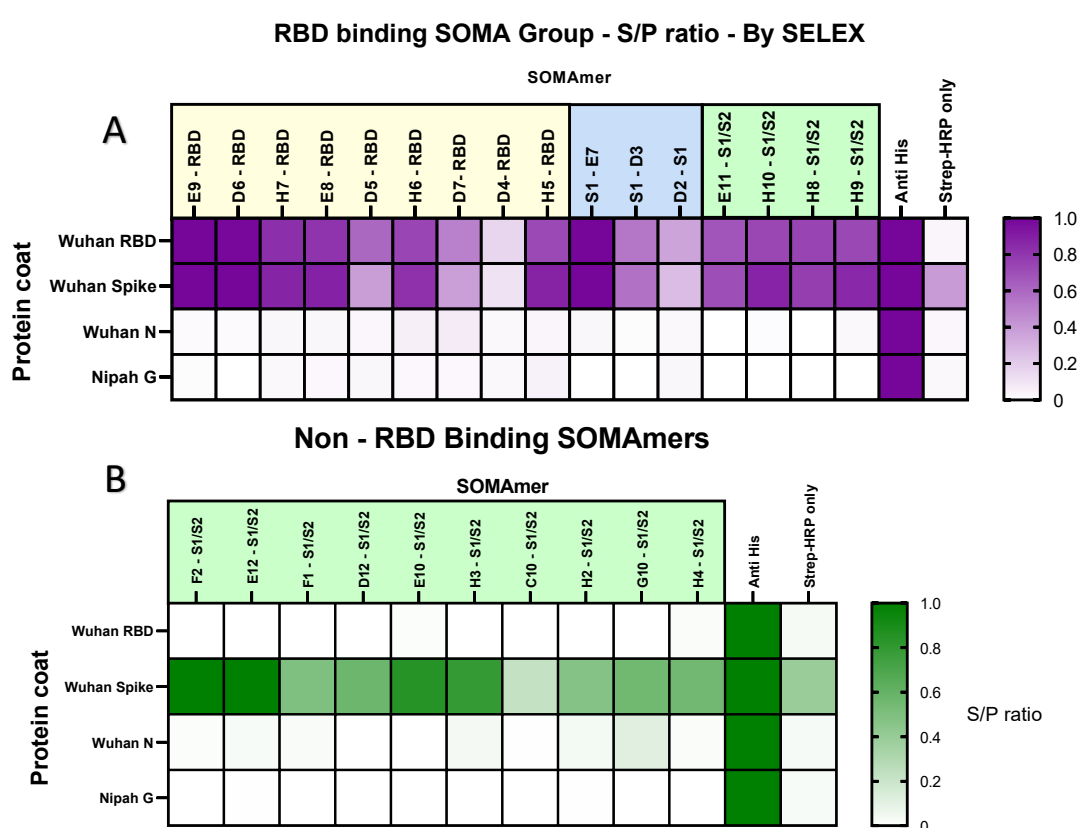


Figure 4.3 : Summary classification of SOMAmers into RBD or non-RBD binding groups.

A. Sixteen RBD-binding SOMAmers identified in Figure 4.2 grouped by the original epitope used in SELEX. SOMAmers displaying binding to both RBD and FL-S were classed as RBD binders, due to the RBD being found in both antigens. SOMAmers binding SARS-CoV-2 Wuhan-Hu-1 RBD, classified as RBD binders are shown in purple.

B. SOMAmers identified in Figure 4.2 as binding to full length spike but not RBD were classed as non-RBD binding SOMAmers, shown in green. For both Figures 4.3A and B, the surrounding box colour on the top x axis shows the spike region used in the selection by SELEX (yellow – RBD), (blue-S1) and (green-S1/S2).

4.2.3 Evaluating SOMAmer binding to SARS-CoV-2 variant RBDs at checkpoints in divergent evolution from 2020-2023.

Since its emergence, the SARS-CoV-2 pandemic has been characterised by ongoing viral evolution driven by selective pressure on the spike protein [122]. This has led to the emergence of multiple variants, including B.1.1.7, P.1, BA.1.59, BA.2, and BA.2.86, among others. In this context, and after identifying SOMAmers capable of binding the Wuhan-Hu-1 spike protein and/or its RBD, we next examined the capacity of each SOMAmer to interact with the RBDs from the following variants: Wuhan-Hu-1 (19A), Alpha (20I), Delta (21A), BA.1 (21K), BA.2 (21L), BA.2.86 (23I), JN.1 (24A), XBB (22F), and EG.5.1 (23F), again using the ELONA assay. As a secondary positive control, the anti-RBD monoclonal antibody CR3022, previously raised against SARS-CoV, was included to demonstrate preservation of an intact and correctly folded RBD target antigen.

Interestingly, the binding patterns of each SOMAmer varied in relation to SARS-CoV-2 phylogenetic evolution, selectively losing or regaining binding (**Figure 4.4**). SOMAmers from the RBD-binding subgroup were screened, along with a single control SARS-CoV-2 nucleocapsid binding SOMAmer (C11), using both additional antigen loading and non-specific binding controls. Anti-His tag mAb binding confirmed that protein loading for each individual RBD in the assays remained high, with non-specific binding remaining low.

Additionally, for the variant BA.1, there was a reduction in signal across all the SOMAmers screened (**Figure 4.4A**). As with pan-variant monoclonal antibodies, SOMAmers rely on the conservation of RBD structure, which can be directly influenced by mutation. In contrast, some SOMAmers selected against the Wuhan-Hu-1 variant lost binding at specific points in the evolution of the SARS-CoV-2 RBD. For example, evolutionary breakpoints in binding appeared in time between Alpha which emerged

in winter 2020 and Omicron BA.1 and BA.2 which arose in winter 2021. Across all three screen replicates, binding responses for some SOMAmers dropped below the positive threshold and did not show binding recovery when screened against later variants.

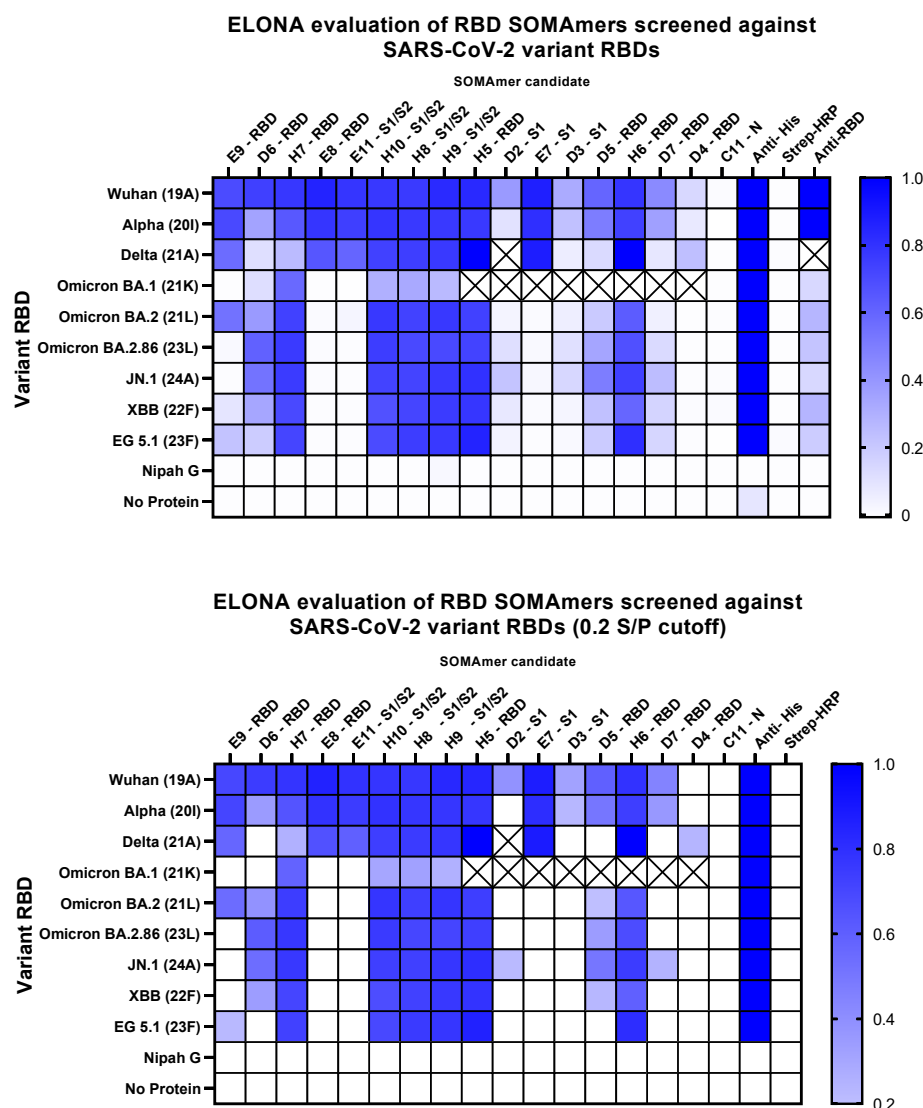


Figure 4.4: Evaluation of Wuhan-Hu-1 RBD-binding SOMAmers binding to SARS-CoV-2 variant RBDs by ELONA.

4A. Heatmap showing the results of three repeat experiments using the ELONA format. SARS-CoV-2 variant RBD proteins (80 nG/well) were coated onto wells and incubated with SOMAmers (80 ng/well). The binding of RBD epitope mapped SOMAmers was evaluated, with colour intensity corresponding to binding signal strength. Signal was collected as raw OD450 with the background then subtracted and expressed as a Signal/Positive ratio (S/P) versus the anti-His mAb control. Crosses show where binding evaluation was not undertaken between RBD and SOMAmers. RBD binding candidates were screened against Wuhan-Hu-1, JN.1, XBB and EG.5.1 RBDs with specific binding controls included, as follows: SOMAmer C11 (Anti Nucleocapsid), Nipah virus G glycoprotein and no protein.

4B. Data presented with a 0.2 S/P ratio cut-off to remove low binders.

To support subsequent description and referencing, each RBD-binding SOMAmer was classified into three subgroups based on binding patterns to SARS-CoV-2 variants, designated as subgroup 1 (pan-variant), subgroup 2 (mutation-limited), and subgroup 3 (intermittent binders) across repeated experiments. The SOMAmers identified and summarised in **Table 4.2** showed strong binding in triplicate repeat experiments across the full panel of variant RBDs (**Figure 4.4**).

As summarised in **Table 4.2**, subgroup 1 SOMAmers, encompassing H7, H10, H8, H9, H5 and H6, showed strong pan-SARS-CoV-2 RBD binding. There were two potential explanations for these shared strong binding responses, either SOMAmers were binding the same epitope on the RBD, which is conserved, or they were binding to different epitopes but strongly enough that the variation in the variants did not affect binding. These subgroup 1 SOMAmers were prioritised as the focus for future studies examining the wider diversity and potential detection of SARS-like betacoronaviruses. Following the ELONA results, subgroup 2 SOMAmers were classified as ‘mutation-limited binders’, i.e. they bound up to a specific event in the evolution of the RBD at which point their binding ability was lost. Within subgroup 2 SOMAmers, the changes in amino acids between Alpha and Omicron BA.1 and BA.2 therefore could be used to indicate residues within RBDs that restrict SOMAmer binding capacity. SOMAmers with a more limited binding range have potential for a variant specific identification. In the context of diagnostic lateral flow test (LFT) capture and detection pairing, a specific binder combined with a pan binder within the test could provide a format for specific virus identification. The third subgroup of SOMAmers were intermittent binders, which were the most unique in their patterns where they lost and regained their binding through virus variant evolution. This group retained the ability to bind early and late evolution variants but lost binding against BA.2 and BA.2.86, XBB and JN.1.

	Pan variant ending (Subgroup 1)	SELEX target	Mutation limited binders (Subgroup 2)	SELEX target	Intermittent binders (Subgroup 3)	SELEX target
1	H7	RBD	E8	S1/S2	E9	RBD
2	H6	RBD	E7	S1	D5	RBD
3	H8	RBD	D4	RBD	D6	RBD
4	H10	S1/S2	E11	S1/S2	D7	RBD
5	H9	RBD	D3	S1	D2	S1
6	H5	RBD				

Table 4.2: Summary of each RBD-binding SOMAmer candidates' classification into subgroups: pan-variant, mutation limited and intermittent binders. Sub-domain region of Wuhan-Hu-1 spike protein used in SELEX referenced for each SOMAmer candidate. Interpretation of binding as > 0.2 Signal/Positive control ratio as in Figure 4.4.

4.2.4 SARS-CoV-2 RBD antigen concentration detection levels by SOMAmers in ELONA

The SOMAmers with higher ELONA binding performance (H6, H7 and H8) were further investigated to explore their potential application in a prototype diagnostic test to detect varying concentrations of recombinant RBD protein in samples using a sandwich ELONA format.

To evaluate the lowest concentration of RBD required to visualise binding, the RBD proteins from SARS-CoV-2 variants Wuhan-Hu-1, BA.2, JN.1 and EG.5.1 were coated onto plates in a dilution range from 25 µg/mL (1250 ng) to 8×10^{-5} µg/mL (0.004 ng) and detected with each SOMAmer at 10 nM.

Across each variant RBD the SOMAmer D5 was the poorest performing over the range of concentrations (**Figure 4.5**). This performance appeared to correlate with accumulated changes in the RBD with later variants showing lower raw binding signal when compared to the SELEX RBD (Wuhan-Hu-1), only detecting antigen at the highest concentration (25 µg/mL) for BA.2, JN.1 and EG.5.1 (**Figure 4.5**). Alternately, H7 performed strongly, displaying signal at an RBD concentration of 0.79 µg/mL (40 ng) for all variants except BA.2 in the ELONA screening (**Figure 4.5**). H6 and H8

performed the strongest over the titration series, and each displayed binding down to concentrations between 0.79-0.25 $\mu\text{g/mL}$ (40 nG to 12.5 ng) for each variant, and 0.08 $\mu\text{g/mL}$ (4 ng) for Wuhan-Hu-1.

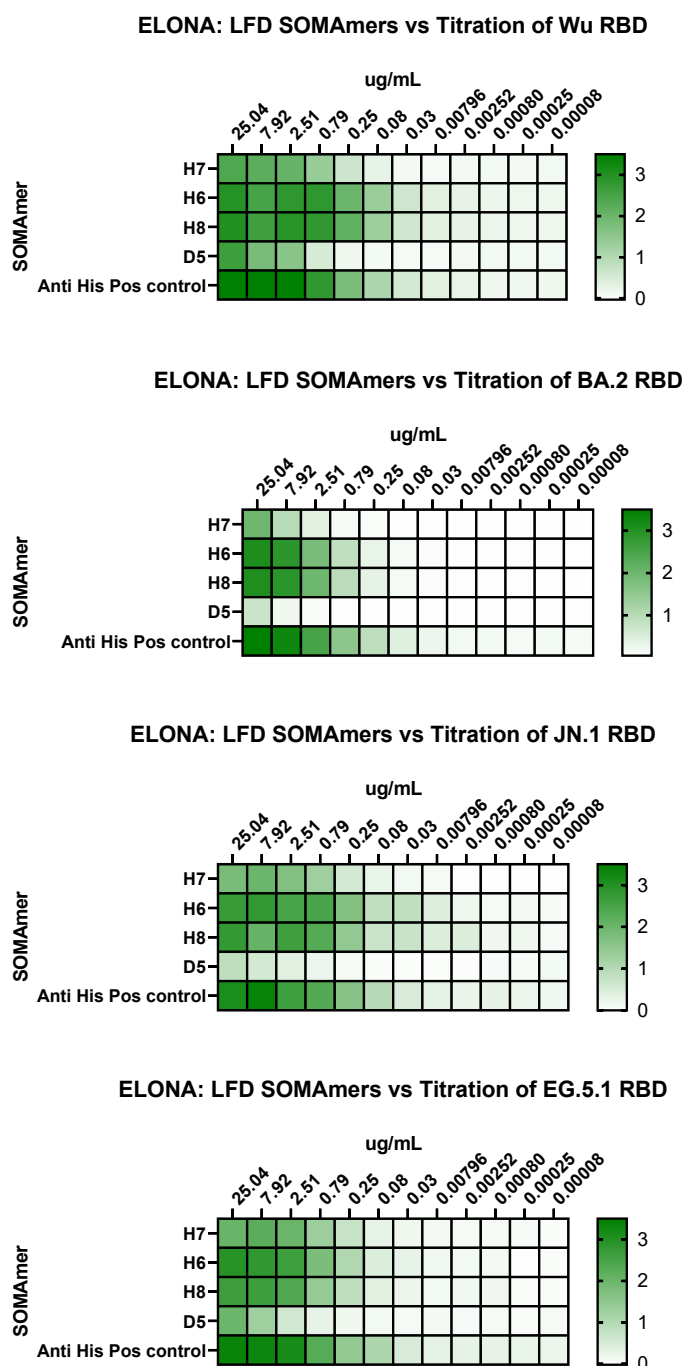


Figure 4.5: Titrations of SARS-CoV-2 variant RBD protein concentration vs RBD binding SOMAmer subset

Lead RBD binding candidates (H7,H6,H8) from subgroup 1 and D5, were evaluated for binding responses against decreasing abundance of SARS-CoV-2 variant RBDs. RBD protein from four selected variants Wuhan-Hu-1 (Winter 2019), BA.2 (Winter 2021), JN.1 (Summer 2023) and EG.5.1 (Winter 2023) were used to coat wells for SOMAmer (10 nM) binding via ELONA. The RBD protein dilution series was made in half log steps (Dilution Factor=3.16). Binding response data averaged from triplicate technical repeats. The colour intensity corresponded to binding signal strength. Signal was collected as raw OD450 with the background then subtracted and expressed as a Signal/Positive ratio (S/P) versus the anti-HIS control.

4.2.5 RBD-binding SOMAmers can detect diverse clades of Sarbecovirus RBDs by ELONA.

Following characterisation of SOMAmer binding to SARS-CoV-2 variant RBDs, a broader range of sarbecovirus RBDs, representing several clades, were used as targets (**Table 4.3**). Reservoirs of sarbecoviruses in bats and other mammals have the potential to cause cross-species transmission. Representative sarbecoviruses used in this evaluation included: clade 1a associated with SARS-CoV, clade 1b associated with SARS-CoV-2, clade 2 non-ACE2 receptor-binding (Southeast Asian), and clade 3 ACE2 receptor-binding (non-Southeast Asian).

Understanding the capacity of SOMAmers to bind across the wider sarbecovirus clades would pave the way for application as diagnostics as part of pandemic preparedness.

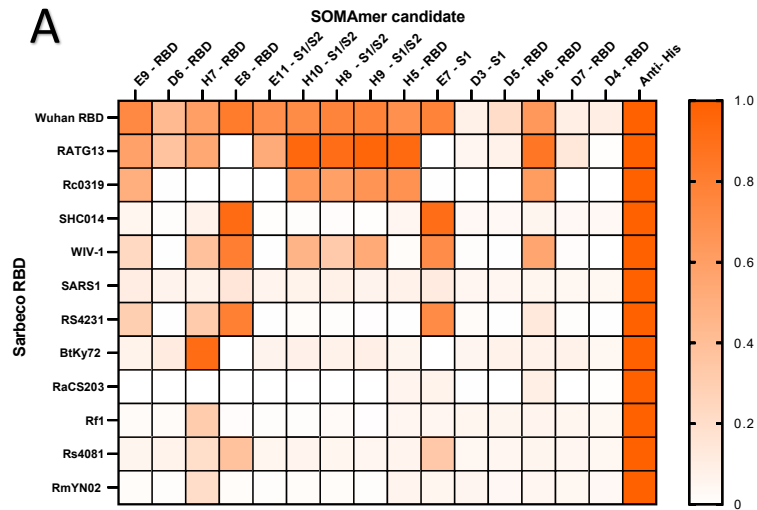
Sarbecovirus	Clade	ACE2 Receptor	% Amino acid identity to Wuhan-Hu-1 RBD
SARS-CoV-2 Wuhan-Hu-1	1b	Human	100
RaTG13	1b	Human	92
Rc-o319	5	Bat, weakly human	74
RsSHC014	1a	Human	79
WIV1	1a	Human	79.5
SARS1	1a	Human	77
RS4231	1a	Human	79
BtKY72	3	Bat	79
RaCS203	1b	Bat	68
Rf1	2	Non-ACE2	71
RS4081	2	Non-ACE2	70
RmYN02	2	Non-ACE2	69

Table 4.3: Sarbecovirus clade definitions and primary host cell receptor use. RBD amino acid identity in comparison with SARS-CoV-2 Wuhan-Hu-1 is shown.

RBD-binding SOMAmers in the library were used for this study to maximise the opportunity to determine binding to diverse clades of sarbecovirus RBDs. SOMAmers fell into different patterns of binding (**Figure 4.6 A and B**). Firstly, several SOMAmers (H7, E8, and E7) retained binding to a broad selection of sarbecoviruses. Secondly, three sarbecoviruses (SARS-CoV-2 Wuhan-Hu-1, RaTG13, Rc-0319 and WIV1) were

recognised by a broad range of SOMAmers. Thirdly, several SOMAmers (D3, D4, D5 and D7) bound only to SARS-CoV-2 variants and were therefore species-specific binders. These characteristics informed the potential choice of SOMAmer pairs for broad coronavirus detection.

ELONA: RBD Binding SOMAmers vs Sarbecovirus RBDs



ELONA: RBD Binding SOMAmers vs Sarbecovirus RBDs (0.2 S/P cutoff)

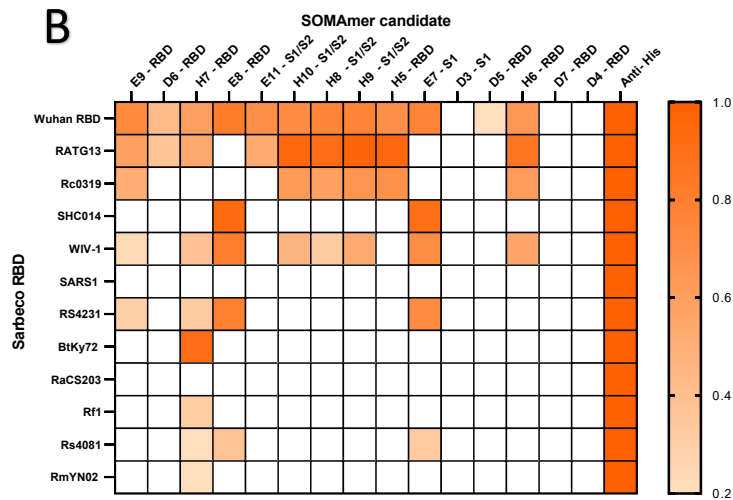


Figure 4.6: Evaluation of RBD SOMAmers binding to Sarbecovirus RBDs by ELONA.

6A. ELONA was used to evaluate the Wuhan-Hu-1 RBD binding SOMAmers (E9-D4) against a subset of Clade 1a,1,3 and 5 Sarbecoviruses RBDs identified within East Asia and mainland China closely related to SARS-CoV-2 RBD. SOMAmers (80 ng/well) were used in ELONA binding to 80 ng/well of viral RBDs. The colour intensity heatmap corresponded to binding responses to RBDs. Binding response data averaged from triplicate technical repeats. Signal collected as raw OD450 was background subtracted and expressed as Signal/Positive ratio (S/P) based on the anti-His control.

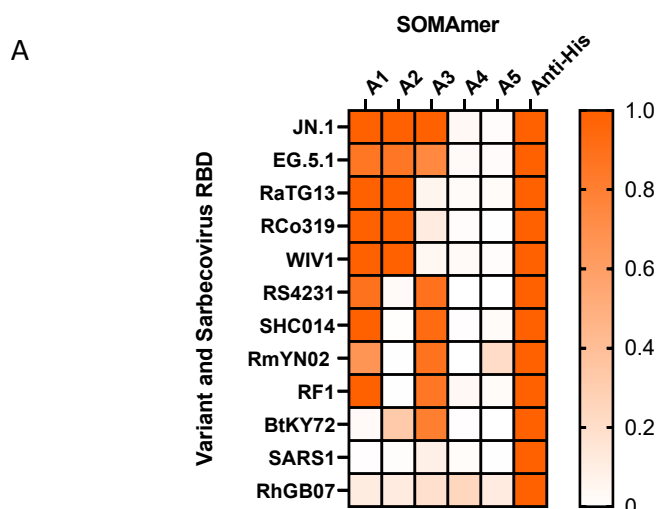
4.2.5.1 Evaluation of neutralising second SOMAmer library binding to Sarbecovirus RBDs.

During the project, alternative SOMAmers derived from the Wuhan-Hu-1 strain RBD or S2 became available. These SOMAmers were part of a separate SOMAmer library and previously shown to have neutralisation potential against multiple SARS-CoV-2 variants [111]. The epitope site within the RBD of one of the second SOMAmer library (SL1111_20, designated A3 in this study) had been determined by competition ELISA with numerous monoclonal antibodies, which had been previously epitope mapped by cryo-EM or X-ray crystallography.

Screening the second SOMAmer library using ELONA against divergent RBDs of various coronaviruses allowed comparison with my SOMAmer library, with the potential to reveal matching binding responses and identification of conserved epitopes. Several SOMAmers from the second library displayed strong binding conservation to some hACE2-utilising RBDs (**Figure 4.9**), including both ancestral relatives and SARS-CoV-2 variants that diversified into multiple lineages under selective mutational pressure.

Comparison of the binding ranges of the second SOMAmer library with my SOMAmer library did not show alignment (**Figure 4.6 and Figure 4.7**). As there was no identical binding pattern between the epitope-mapped Oxford SOMAmer (SL1111_20, A3) and any of the SOMAmers in my library, then it could not be concluded that there was a shared epitope. Therefore, the location of epitopes used by my SOMAmer library could not be inferred by comparison with A3.

ELONA Screen of Oxford SOMAmers vs Sarbecovirus RBD proteins



ELONA Screen of Oxford SOMAmers vs Sarbecovirus RBD proteins (0.2 S/P cutoff)

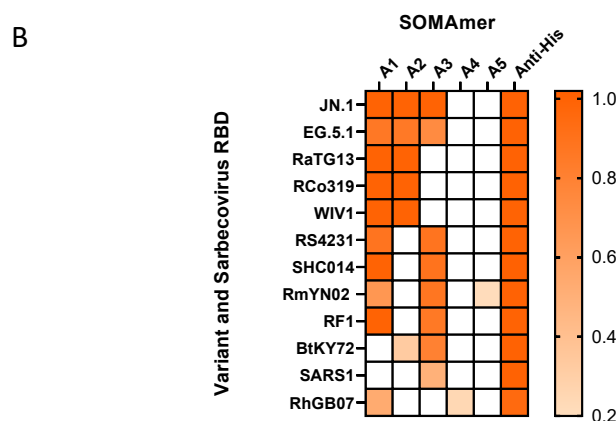


Figure 4.7: Evaluation of neutralising second SOMAmer library binding to Sarbecovirus RBDs by ELONA.

7A. Heatmap of ELONA assay using alternate SOMAmer candidates selected vs SARS-CoV-2 Wuhan-Hu-1 with neutralising capacity. SOMAmers (80 nG/well) were used in ELONA binding to 80 nG/well of total RBD. The colour intensity heatmap corresponded to binding responses to RBDs. Binding response data averaged from triplicate technical repeats. Signal collected as raw OD450 was background subtracted and expressed as Signal/Positive ratio (S/P) based on the anti-His mAb control.

7B. Data presented with 0.2 S/P ratio to remove SOMAmers with low binding.

4.2.6 Evaluation of SOMAmer binding to wider Beta-CoV, Alpha-CoV species full length spike proteins.

As demonstrated above, subsets of SARS-CoV-2 Wuhan-Hu-1 selected SOMAmers were capable of binding in ELONA to firstly, SARS-CoV-2 Wuhan-Hu-1 RBD/spike protein (**Figure 4.3**), secondly, to SARS-CoV-2 variants (**Figure 4.4**) and then to some wider sarbecoviruses (**Figure 4.6**). To evaluate the potential broader range of SOMAmer binding to coronavirus species more diverse than SARS-CoV-2 and related sarbecoviruses, other full length spikes were used in additional binding evaluations.

The range of viruses investigated included human circulating seasonal species (Alpha-CoVs: 229E, NL63, Beta-CoV: HKU1 and OC43), Beta-CoVs of pandemic concern (MERS-CoV, SARS-CoV) and those of veterinary concern to farmyard animal species, e.g. porcine respiratory CoV (PRCV). These viral species utilise a variety of host cell receptors compared to SARS-CoV-2, therefore it would be expected that their RBDs would be diverse, however regions of S1 and S2 are more conserved at the amino acid level [123].

Again, to maximise the potential for binding of SOMAmers to this more diverse range of coronaviruses, the panels of SOMAmers chosen for this study include those that bind to RBD, and those that bind to spike protein outside of the RBD. To complement this approach, full length spike proteins were used as the viral targets.

Evaluation of SARS-CoV-2 Wuhan-Hu-1 selected RBD- and non-RBD binding SOMAmers indicated no cross-reactive binding was observed across the spike proteins evaluated, except between SARS-CoV-2 and SARS-CoV (**Figure 4.8**).

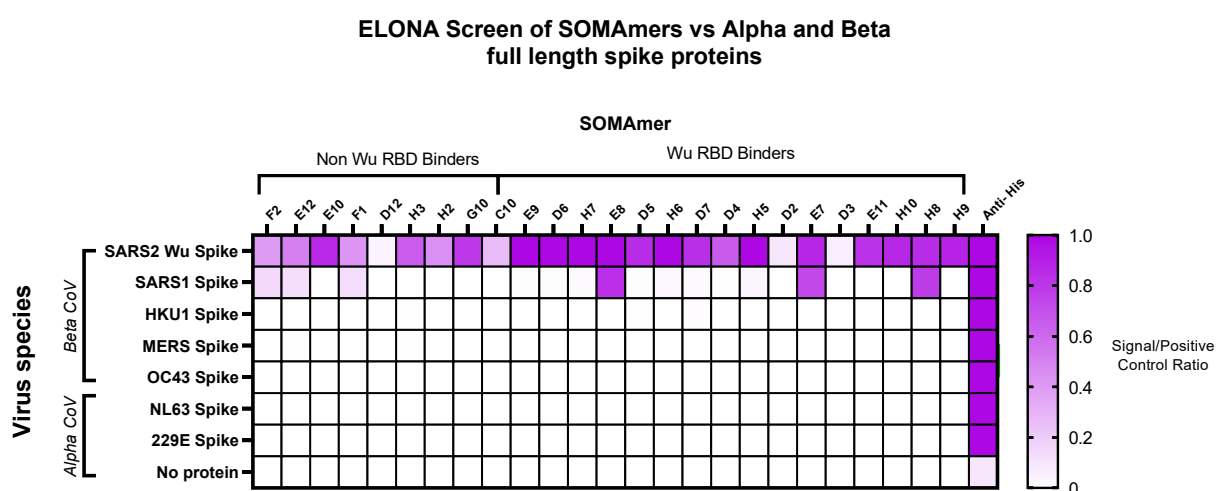


Figure 4.8: Evaluation of SOMAmer binding to wider Alpha and Beta CoV full length spike proteins by ELONA.

SOMAmers from the subgroups of RBD and non-RBD binders were screened against FL-spikes of Alpha and Beta Genus CoVs by ELONA.

SOMAmers (80 ng/well) were used in ELONA binding to 80 ng/well of FL-spike proteins. The colour intensity heatmap corresponded to binding responses to FL-spikes. Binding response data averaged from *triplicate* technical repeats. Signal collected as raw OD450 was background subtracted and expressed as Signal/Positive ratio (S/P) based on anti-His mAb control. Colour indicates the strength of binding response.

4.2.6.1 Evaluation of neutralising second SOMAmer library binding to wider Beta-CoV and Alpha-CoV species full length spike proteins.

Alternative, epitope-mapped SOMAmers derived from the Wuhan-Hu-1 strain, previously shown to have neutralisation potential against multiple variants were available as part of a separate SOMAmer library [111]. These second library SOMAmer were previously shown to bind to SARS-CoV-2 variants and some wider sarbecoviruses (**Figure 4.7**). Evaluation of SOMAmer binding against full length spike proteins derived from a wider range of beta- and alphacoronaviruses would establish the breadth of potential as detection molecules.

SOMAmers from the second library (A1 to 5, demonstrated as neutralising to SARS-CoV-2 variants) were evaluated for binding against wider CoV species full length spikes (**Figure 4.9**). Surprisingly, cross-reactive binding was observed for SOMAmers

A1, A3, A4, and A5 against seasonal H-CoV 229E and HKU1 RBDs above the cutoff threshold of 0.2 S/P. Low levels of binding of SOMAmers were found against OC43, SARS1 and PRCV RBDs, falling below the positive classification for signal strength. As above, comparison of the binding ranges of the second SOMAmer library with my SOMAmer library did not show alignment (**Figure 4.8 and Figure 4.9**). Again therefore, the location of epitopes used by my SOMAmer library could not be inferred by comparison with A3.

ELONA Screen of Oxford SOMAmer and LFT candidates vs Alpha, Beta FL Spike proteins

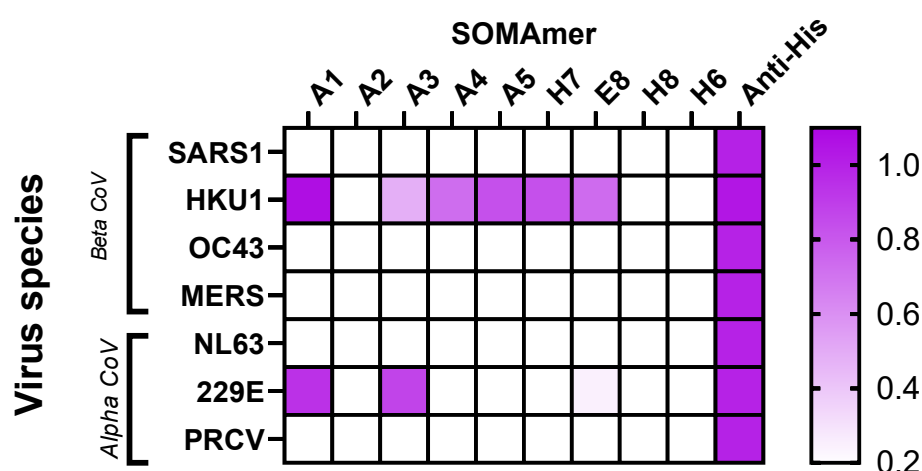


Figure 4.9: Evaluation of neutralising Oxford Wuhan-Hu-1 SOMAmers binding to Alpha and Beta FL-Spikes by ELONA.

SOMAmers (80 ng/well) were used in ELONA binding to 80 ng/well of FL-spike proteins. The colour intensity heatmap corresponded to binding responses to FL-spikes. Binding response data averaged from triplicate technical repeats. Signal collected as raw OD450 was background subtracted and expressed as Signal/Positive ratio (S/P) based on anti-His mAb control. Colour indicates strength of binding response.

4.2.7 Evaluation of SOMAmer binding to Gamma-CoV full length spike protein

Although SOMAmers only showed low levels of binding to the betacoronavirus SARS-CoV (**Figure 4.8**), an evaluation was carried out to investigate whether SOMAmers could detect an even more diverse coronavirus from the gammacoronavirus group (avian infectious bronchitis virus, IBV). For this study, full length spike proteins were used as target antigen for SOMAmers which bound outside the RBD region. IBV spike has only 31% amino acid homology with SARS-CoV-2 Wuhan-Hu-1 spike protein, and IBV uses a separate host cell receptor molecule (α 2,6 linked sialic acid residues [124]) compared with ACE-2 for SARS-CoV-2. Therefore, SOMAmers binding to the RBD region were not chosen for this study as epitopes were less likely to be conserved compared with full length spike protein targets. As part of this experiment, non-RBD binding SOMAmers evaluation against a range of alphacoronavirus (porcine respiratory coronavirus (PRCV)), and betacoronaviruses (OC43, SARS-CoV-2) were repeated (as above in **Figure 4.8**). There was no SOMAmer binding to this wider range of coronaviruses indicating likely loss of conserved epitopes in the spike proteins (**Figure 4.10**).

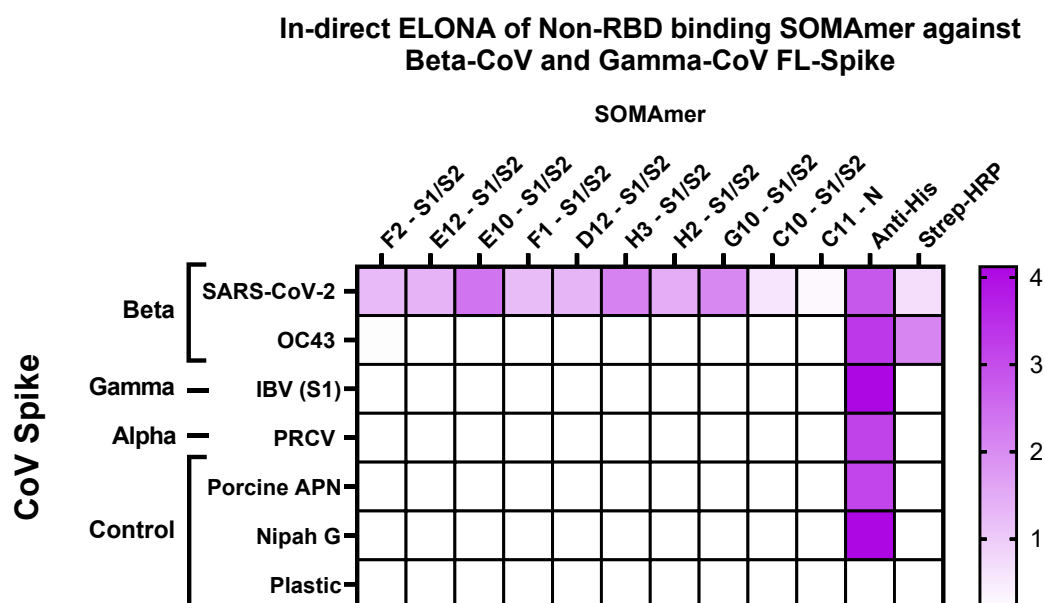


Figure 4.10: Evaluation of non-RBD SOMAmers binding to Alpha, Beta and Gamma Spike by ELONA

SOMAmers binding to non-RBD regions, screened against divergent coronavirus species showed no cross-reactive binding to Alpha (porcine respiratory coronavirus (PRCV), Beta (OC43, SARS-CoV-2) and Gamma (avian infectious bronchitis virus (IBV)). SOMAmers (80 ng/well) were used in ELONA binding to 80 ng/well of FL-spike proteins. The colour intensity heatmap corresponded to binding responses to FL-spikes.

Binding response data averaged from triplicate technical repeats.

Signal collected as raw OD450 was background subtracted and expressed as Signal/Positive ratio (S/P) based on anti-His mAb control. Non-coronavirus antigen controls for non-specific binding, Porcine APN as an entry receptor, Nipah virus G protein and no protein control showed no background binding by SOMAmers within ELONA.

4.2.7 Summary diagrams of cross-reactive binding by SARS-CoV-2 Wuhan-Hu-1 selected SOMAmers against SARS-CoV-2 variants and other sarbecovirus RBDs.

An overview of the binding patterns of SOMAmers to a range of coronaviruses by ELONA, summarising results from previous studies (Figures 4.4, 4.6, and 4.7) is shown below (Figure 4.11).

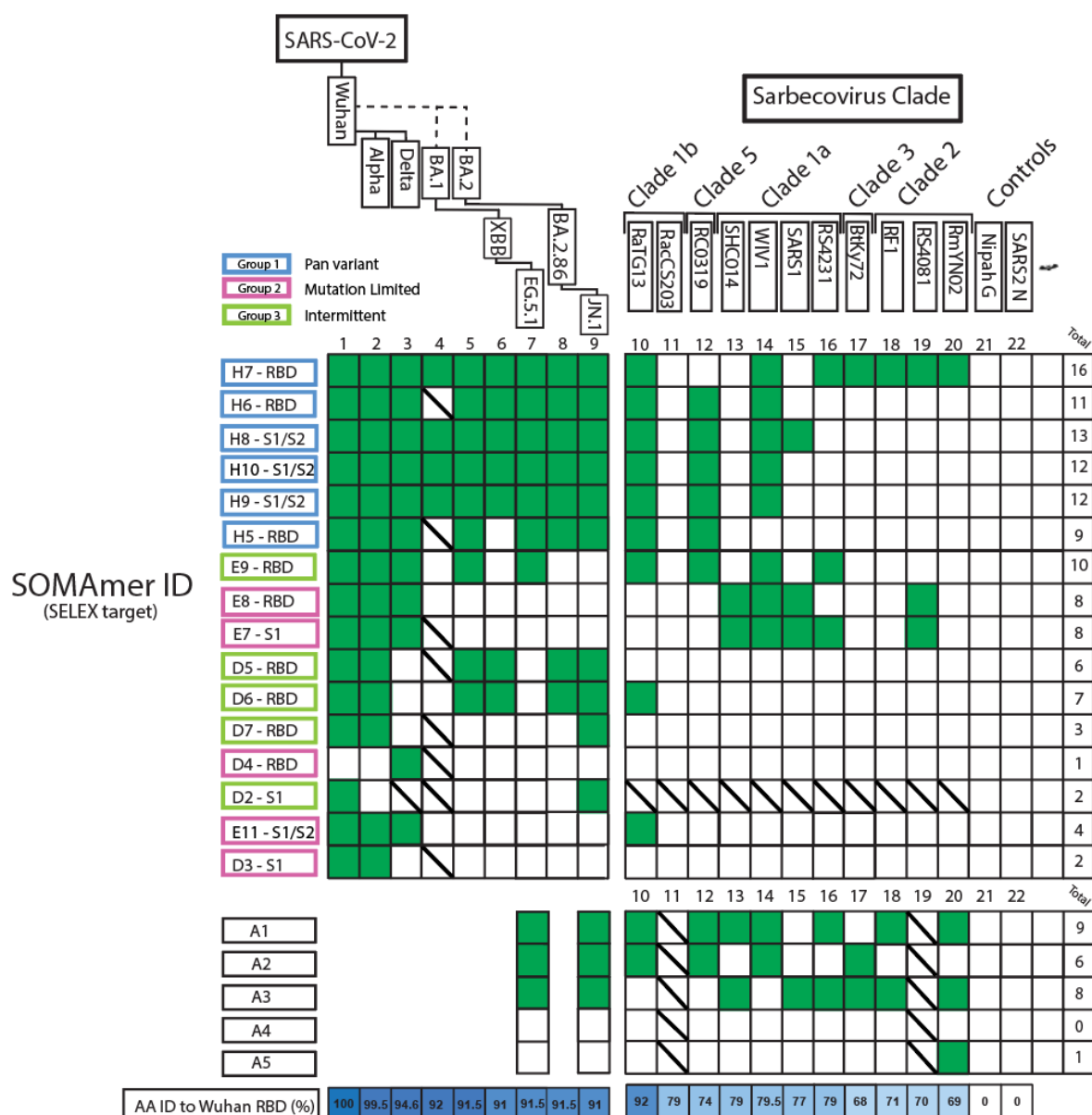


Figure 4.11: Summary diagram of the ELONA screening of RBD-binding SOMAmers to the RBDs of SARS-CoV-2 variants and related sarbecoviruses.

The illustration shows patterns of SOMAmer binding to coronavirus RBDs showing numbers of viruses bound (total column). Data derived from Figures 4.4, 4.6, and 4.7. The cutoff threshold for binary positive/negative classification was 0.2 S/P ratio based on anti-His mAb positive control binding.

The amino acid identity of each RBD expressed as a percentage relative to SARS-CoV-2 Wuhan RBD is displayed on the X axis of the diagram. Binding responses shown as green boxes indicate binding above 0.2 S/P ratio and white boxes indicate low/no binding (below 0.2 S/P ratio). Binding response data averaged from triplicate technical repeats from triplicate experiments.

Each binding interaction within ELONA used this cutoff value to classify responses quantitatively as a positive or negative. Diagonal dashes indicate that evaluation between RBD and SOMAmer was not performed.

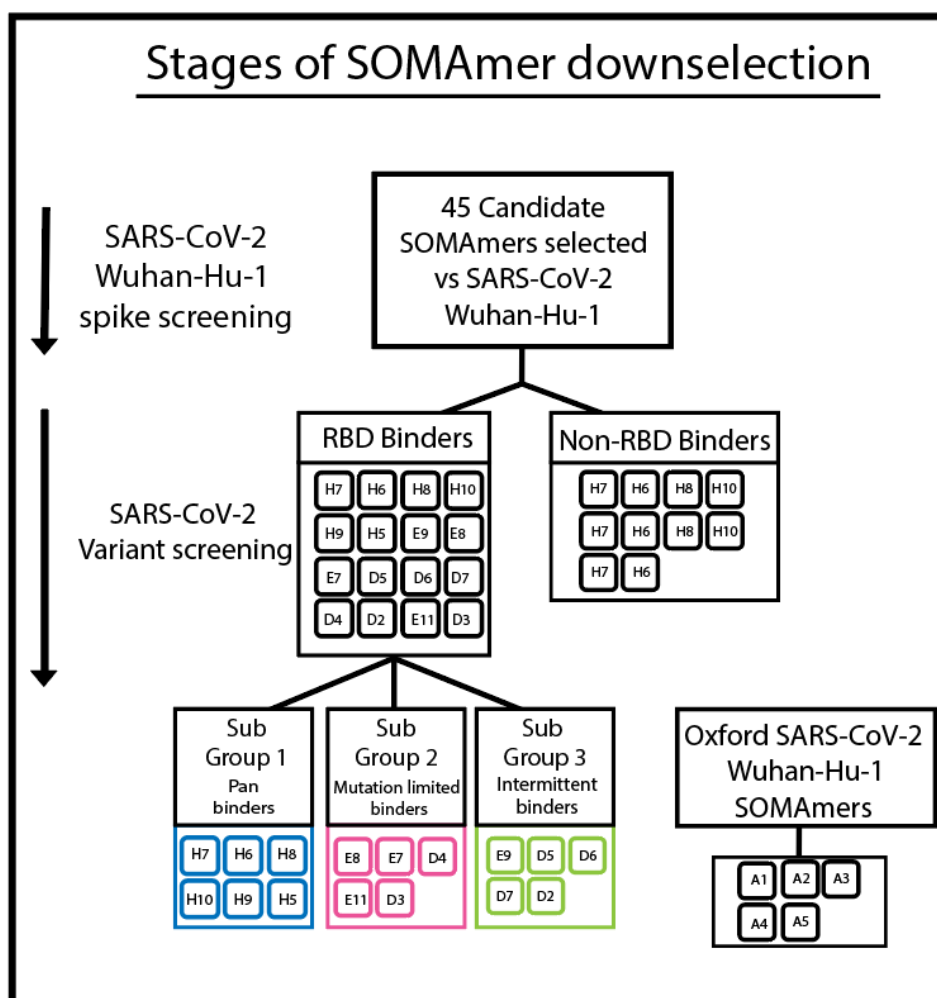


Figure 4.13: Diagram illustrating SOMAmer classification and down selection into binding groups.

The initial library of 45 SARS-CoV-2 Wuhan-Hu-1 RBD: S1: S1/S2 selected SOMAmers was classified into RBD or non-RBD binders (data from **Figure 4.2**). The RBD binding group of SOMAmers was further evaluated (data from **Figure 4.4**) for binding patterns to SARS-CoV-2 variants and fell into three sub-groups. (Pan variant – blue) (Mutation limited – pink) and (Intermittent binders - green). Non-RBD binders were evaluated for binding against Alpha, Beta and Gamma coronavirus species. SOMAmers (19 in total) which fell below the binding cut off value of 0.2S/P ratio were not taken forward for further evaluation.

SOMAmers fell into three distinct patterns of binding response against SARS-CoV-2 variant RBDs (**Figures 4.4 and 4.12**). These were subgroup 1 ‘pan-variant’ (outlined with a blue box), where SOMAmer binding was maintained across evolutionary variants, subgroup 2 ‘mutation-limited’ (outlined pink) where SOMAmers bound along the evolution path of variants but stopped at a discrete point, and subgroup 3

‘intermittent’ (outlined with a green box) where SOMAmers bound along the evolution path of variants, stopped binding, then resumed binding at a later stage of evolution. Using identical conditions, SOMAmers were screened against a single concentration (1.6 µg/mL, 80 nG/well) of RBD proteins from sarbecoviruses clades 1a, 1b, 3 and 5, representing viral species that share sequence similarities with SARS-CoV and SARS-CoV-2 (**Figure 4.6**).

Subgroup 1 SOMAmers, classified as SARS-CoV-2 pan-variant binders used in this study, included H5, H6, H7, H8, H9, and H10 (**Figure 4.4 and 4.12**). From our data there appeared to be some epitopes that supported binding of SOMAmers across various sarbecovirus RBDs. Two SOMAmers classified in subgroup 1 (H7 and H6) retained their binding to sarbecovirus RBDs; however, others, such as H8, H10, H9, and H5, lost binding to RS4231 (**Figure 4.6 and 4.11**).

Subgroup 2 SOMAmers, which displayed mutation-limited binding to SARS-CoV-2 variants (E8, E7, and E11), displayed divergent sarbecovirus responses (**Figure 4.6 and 4.11**): E8 and E7 retained binding, whereas E11 lost binding to WIV-1 and RS4231. Subgroup 3 SOMAmers, which exhibited intermittent binding to SARS-CoV-2 variants (E9, D6, D3), were similarly mixed in their sarbecovirus binding performance (**Figure 4.6 and 4.11**): E9 retained binding to RaTG13, WIV-1, and RS4231, whereas D6 and D3 lost binding to WIV-1 and RS4231 while retaining binding to RaTG13.

To provide a more detailed chronological summary, I also provide an overview of SARS-CoV-2 evolution (as shown in **Figure 4.1**) overlayed with the binding ranges of Subgroups 1, 2 and 3 SOMAmers (**Figure 4.13**). Subgroup 1, pan-variant binding SOMAmers recognised all variants tested, suggesting the binding site is conserved. In contrast, the binding capacity of subgroup 2 mutation-limited SOMAmers ceased at the evolutionary time before Omicron, suggesting the binding site is lost. Lastly,

subgroup 3, the intermittent binding SOMAmers, are thought to bind to epitopes that appear to be lost, but which then reappear in subsequent variants.

The precise locations of the epitopes that lead to these different patterns of binding remained undetermined at this stage, but distinct binding patterns relating to RBD mutation were visible. Interestingly the number of SOMAmers in each subgroup was larger in the pan-variant group as shown in blue shading in **Figure 4.13**, compared with other subgroups, suggesting that the SOMAmer library might have a hidden predisposition to conserved epitopes.

Cumulative confirmed cases - World (January 2020 - 2023)

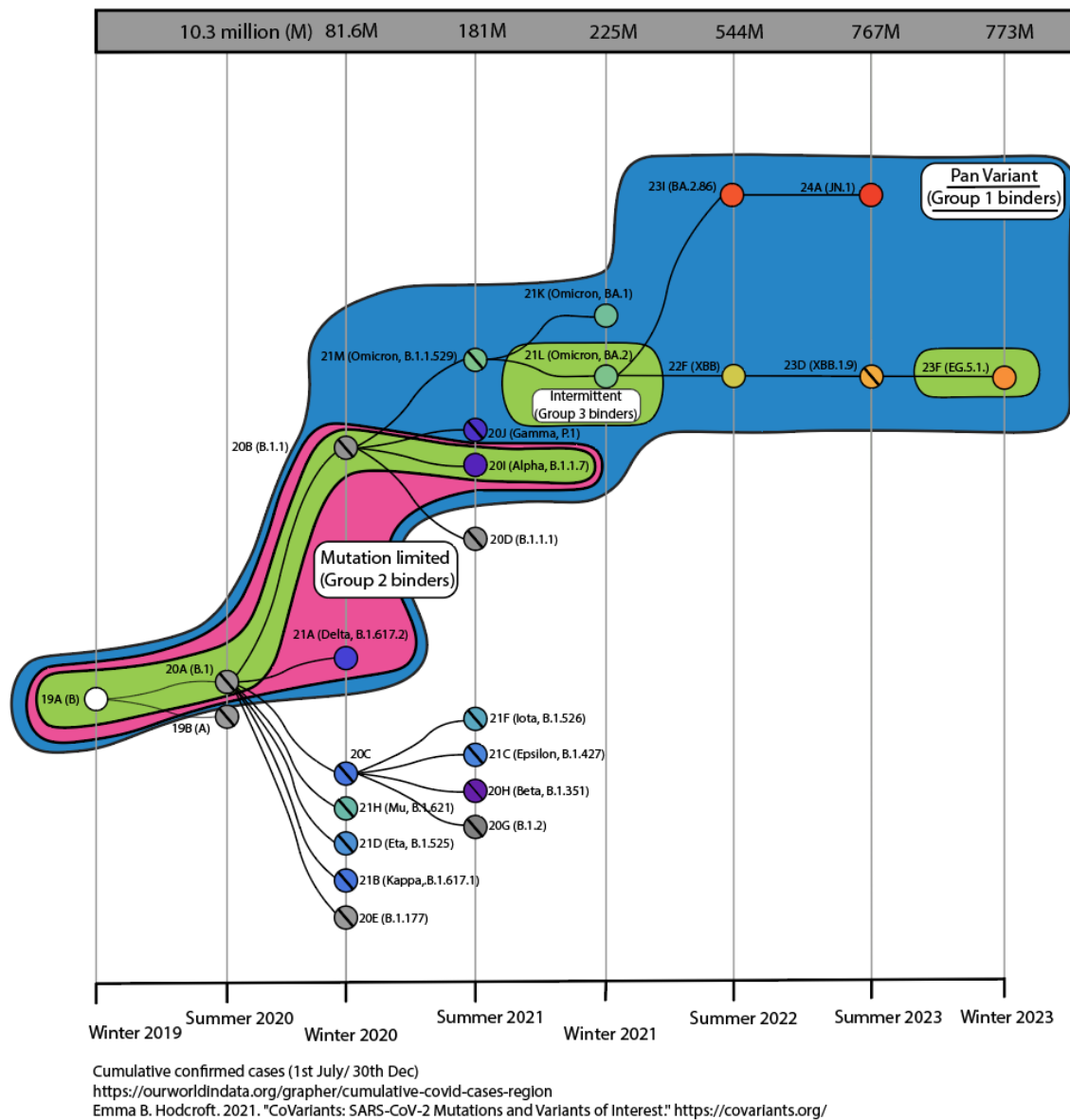


Figure 4.13: Binding ranges of SARS-CoV-2 RBD SOMAmer subgroups mapped to phylogenetic tree of SARS-CoV-2 variants by ELONA.

Diagram showing binding ranges by ELONA evaluations summarised in Figures 4.4A/B and 4.11. Blue shadow showing subgroup 1 (pan-variant binders), pink shadow showing subgroup 2 (mutation limited binders), and green shadow showing subgroup 3 (intermittent binder).

4.2.8 Summary diagram of cross-reactive binding by SARS-CoV-2 Wuhan-Hu-1 selected SOMAmers against divergent coronaviruses of Alpha, Beta and Gamma CoV

Cross-reactive binding by SARS-CoV-2 Wuhan-Hu-1 selected SOMAmers against full length spike proteins of divergent coronavirus of Alpha, Beta and Gamma CoV in addition to SARS-CoV-2 variants and Sarbecovirus Clade 1a, 1b is summarised in

Figure 4.14 (derived from data in **Figures 4.8, 4.9 and 4.10**).

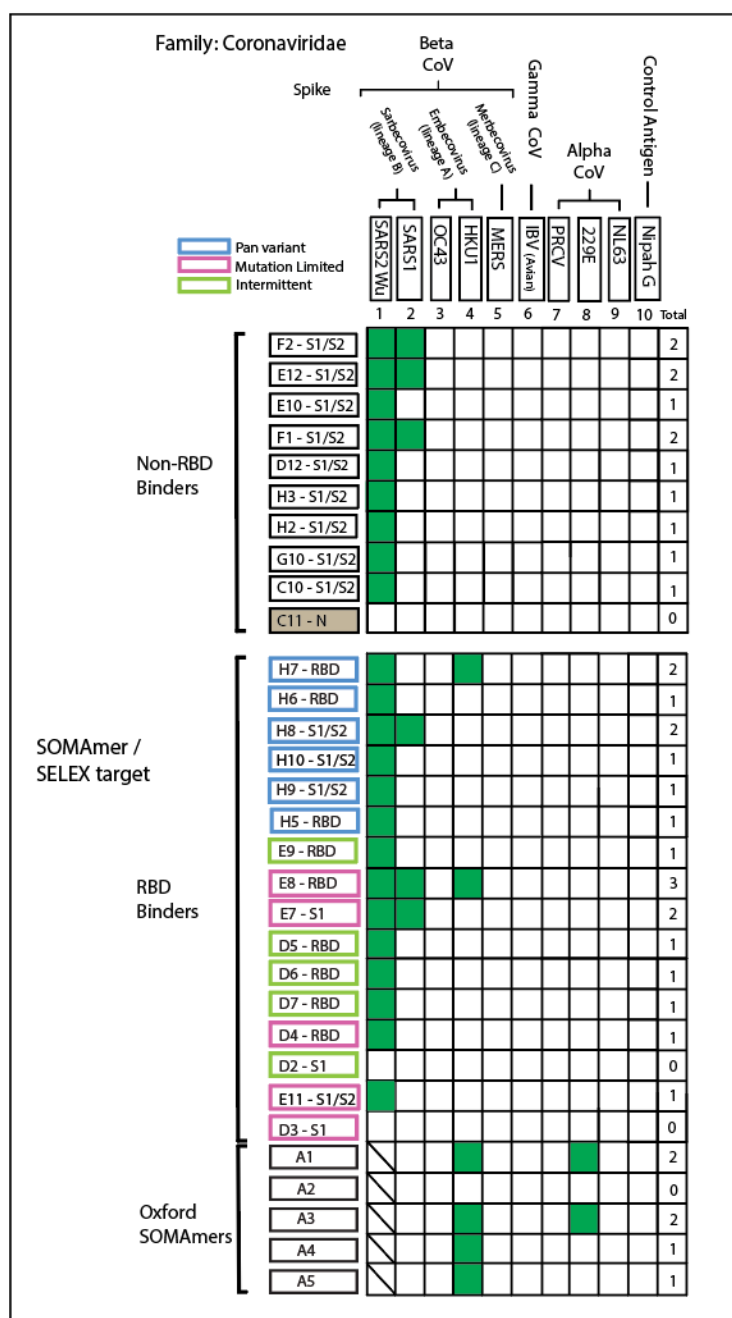


Figure 4.14: Summary diagram of ELONA screening of SOMAmers to FL-Spike of Alpha, Beta and Gamma-CoV genera .

The illustration shows patterns of SOMAmer binding to coronavirus FL-spike proteins (derived from Figures 4.8, 4.9 and 4.10). The cutoff threshold for binary positive/negative classification was 0.2 S/P ratio based on the anti-His mAb positive control. Binding response data averaged from triplicate technical repeats from triplicate experiments.

Binding responses were expressed as follows: green boxes to indicate binding above 0.2 S/P ratio and white boxes to indicate low/no binding (below 0.2 S/P ratio). Diagonal dashes indicate that evaluation between RBD and SOMAmer was not performed.

Non-RBD binding SOMAmers from the original library showed that binding was limited to SARS-CoV-2 and SARS-CoV. In contrast, SOMAmers H7 and E8 which bound the RBD region, also bound to HKU1 FL-spike as did several of the SOMAmers from the second SOMAmer library. In addition, two of the Second library SOMAmers bound to 229E FL-spike.

4.2.9 – Identifying SOMAmers to be used in diagnostic applications – Capture and detection roles within a prototype lateral flow test

Following the conclusion of ELONA screening of candidate SOMAmers (summarised in **Figure 4.11**) the following SOMAmers: H10, H9, H8, H7, H5, E7, E8, and D4 were shown to have broad and strong binding capacity in ELONA. These SOMAmers were selected for investigation of potential non-competitive binding to viral target, which would enable capture and detection pairings to be defined for evaluation in prototype LFT formats. SOMAmer C11, selected against the SARS-CoV nucleocapsid, was taken forward as a control for non-specific binding. At this point, further investigation of binding epitopes and the design of non-competitive binding pairs focused on SARS-CoV-2 RBD as the target antigen.

4.3 Discussion

Ongoing evolutionary pressure drives viruses to evade host immunity while maintaining efficient receptor binding and viral fitness. Viral antigen diversity is a barrier to broadly reactive vaccines, molecular recognition elements for diagnostics, and therapeutics against coronaviruses. Finding single-stranded DNA SOMAmers that can continue to bind, despite the effects of evolution, is a challenging objective. Evolution driven by mutation influences primary amino acid sequence, which subsequently affects conformation, charge, and hydrophobicity of proteins relevant to SOMAmer binding. The levels of antigenic diversity within a given viral species are referred to as antigenic drift, a stepwise change within a lineage circulating in a host population.

This study employed a diversity of viral RBDs and full-length spike proteins within a single species, as well as inter-subgenus diversity between sarbecoviruses comparable to SARS and SARS-CoV-2. Beta-CoV species include those infecting humans during seasonal periods (OC43, 229E, and NL63), and coronaviruses that have remained endemic but possess pandemic potential (SARS-CoV, MERS-CoV). Full-length spike proteins of CoV species of veterinary importance were also evaluated for cross-reactive binding, including infectious bronchitis virus (IBV) in avian species and porcine respiratory coronavirus (PRCV) in pigs.

The evaluation strategy was designed to examine the breadth of viral binding by the SOMAmer library, starting with SARS-CoV-2 Wuhan-Hu-1 and expanding the range of target antigens out to SARS-CoV-2 variants, wider sarbecoviruses and further alpha-, beta- and gammacoronaviruses. Starting with an extensive SOMAmer library, the aim was to select strongly binding viable candidates for two complementary roles in the sandwich ELONA-based diagnostic: 1) a wide-ranging binder and 2) a variant- or species-specific binder.

Due to the logistical challenges associated with cloning, expression, and purification of full-length spike proteins, and the greater availability of recombinant RBDs, the screening was refined to focus solely on RBD-binding SOMAmers. Despite this, the representation of twenty-seven surface viral glycoproteins provided a diverse picture of SOMAmer binding performance across the family, achieving the objective of diverse coronavirus screening for SARS-CoV-2 Wuhan-Hu-1-selected SOMAmers.

The aim of this chapter was to examine the coronavirus diversity and strength of SOMAmer binding by ELONA. This information was required to select SOMAmers to take forwards as non-competing pairs for capture and detection roles in prototype lateral flow test development.

The binding of SOMAmers to the SARS-CoV-2 Wuhan-Hu-1 RBD and spike protein was studied to validate the SELEX targets (RBD, S1, S1/S2). SOMAmers from RBD SELEX specifically bound to the RBD (**Figures 4.2, 4.3A**), while those from S1 or S1/S2 SELEX also bound to regions outside the RBD (**Figures 4.2, 4.3B**). Out of 45 SOMAmers, 26 were identified as either RBD binders (16 SOMAmers) or non-RBD binders (10 SOMAmers). Nineteen SOMAmers showed inadequate binding signals in the ELONA assay and were excluded from future studies. The validation of binding to the SARS-CoV-2 RBD and spike protein confirmed the SOMAmer library data,

facilitating further studies on various SARS-CoV-2 variants. ELONA was used to screen the 16 RBD binders against RBDs from 7 key SARS-CoV-2 variants from winter 2019 to winter 2023, leading to three SOMAmer subgroups summarised in **Table 4.2**. Pan-variant binders of subgroup 1 retained their binding ability across all variant RBDs evaluated, which arose through mutation over a 3-year period of the pandemic. Over this period, an additional 773 million confirmed cases of COVID-19 were reported globally, providing extensive opportunities for mutation to occur. This longevity of binding is a useful feature for the application of these SOMAmers as components of diagnostic assays. Despite the spike protein being subject to the most evolutionary pressure of all CoV structural proteins, it can be assumed that this SOMAmer subgroup is binding to a region of the RBD that has remained conserved in response to evolutionary pressure.

In contrast, mutation-limited SOMAmers within subgroup 2 (E8, E7, D4, E11, D3) showed that binding ability was lost as a result of significant antigenic drift away from Wuhan-Hu-1 residues, such that binding regions were no longer present. This change occurred in variants that arose between summer and winter 2021, so binding was present up to the Delta and Alpha variants, but not for Omicron BA.1 and BA.2. A comparison of RBD amino acid sequence changes across these SARS-CoV-2 variants (**Figure 4.15 B and C**) shows several substitutions between these variants which may be associated with a change in secondary and tertiary RBD structure and loss of binding by this group of SOMAmers.

Alignment of SARS-CoV-2 RBD amino acid sequences.

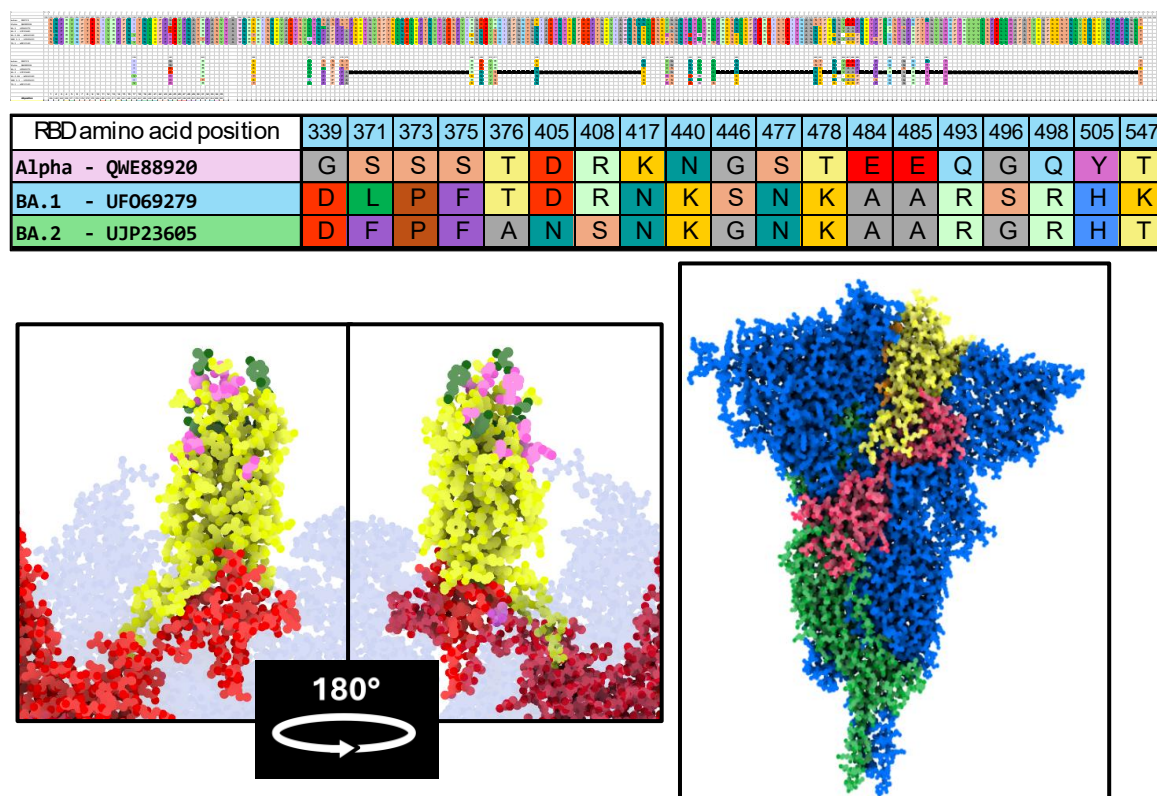


Figure 4.15: Potential RBD residues responsible for loss of mutation-limited SOMAmers (binding occurred with SARS-CoV-2 Alpha (B.1.1.7), but not BA.1 and BA.2 variants).

A - Representation of RBD amino acid sequence alignments of SARS-CoV-2 variants.

B - Highlighted amino acid residues identified as diverging between Alpha (B.1.1.7), BA.1 and BA.2 variants.

C - Protein imager representations of diverging RBD residues identified in Alpha, BA.1 and BA.2 (yellow – RBD, red - S1, dark green - ACE2 contact residues, pink – location of amino acid variation between Alpha (B.1.1.7), BA.1 and BA.2).

D - Representation of SARS-CoV-2 spike glycoprotein showing location of RBD (yellow – RBD, red-S1, green- S2, blue – second and third spike monomers)

The third subgroup of SOMAmers were intermittent binders, which were the most unique in their patterns where they lost and regained their binding through virus variant evolution. This group retained the ability to bind early and late evolution variants but lost binding against BA.2 and BA.2.86, XBB and JN.1. This is not understood, however, if the epitopes for SOMAmers were known it may be possible to assess if binding regions in RBDs were re-created through mutation. Comparison of RBD amino acid sequences of Wuhan-Hu-1 and variant JN.1 does not show obvious reversion; therefore, this feature of regained binding is likely due to an alternative structural change. Of note, several SOMAmers (D3, D4, D5 and D7) bound only to SARS-CoV-2 variants and were therefore species-specific binders. ELONA has been used here as a screening assay to evaluate the strength and range of SOMAmer binding to various coronavirus spike proteins and RBDs to enable selection to take forward to LFD prototype development. However, potential diagnostic assays based on ELONA utilising aptamers as detection recognition elements have also been demonstrated to potentially operate at a clinically-relevant viral load [125], [126].

The limits of detection of a small group of RBD-binding SOMAmers (H6, H7, H8 and D5) was evaluated by ELONA using a wide RBD protein concentration range of 25 µg/mL to 8×10^{-5} µg/mL (1250 nG to 0.004 ng) (**Figures 4.5, 4.6, and 4.7**). SOMAmers H6 and H8 showed binding to Wuhan-Hu-1 RBD down to 0.08 µg/mL (4 ng total). An observable trend was that D5 and H7 displayed weaker binding at lower antigen concentrations than H6 and H8 across all variant RBDs tested (Wuhan-Hu-1, BA.2, JN.1, EG.5.1). These differences may reflect variations in binding affinity between SOMAmer and antigen, potentially driven by SOMAmer structure and sequence variation.

The limit of detection data generated in this study was compared with other published studies to provide context (**Table 4.4**), however the variation in experimental design between aptamer-based ELONA studies makes direct comparison challenging. In addition, RBD as a target molecule used in this SOMAmer study is smaller at (25 kDa) than full length spike (180 kDa) used in the Gupta et al, 2022 and David and Kanayeva, 2022 papers. However, the limits of detection by SOMAmers H6 and H8 was comparable with data derived from these other published studies. It is challenging to align recombinant viral protein concentrations with known virus concentrations or Ct values to determine limit of detection measurements. Virus concentration is measured by plaque assay caused by infection of cells with an infectious virion. Within a virus sample there will be viral proteins not associated with infectious particles, such as non-infectious defective particles or secreted material which over-represent protein concentration relative to infectious virus particle concentration. Therefore, it is difficult to assign a physiologically-relevant concentration range of viral protein analogous to potential viral loads in a patient sample.

To establish if an assay is sufficiently sensitive in a clinical setting, a range of clinical samples need to be examined and compared with the gold standard RT-qPCR test. This was achieved by Gupta et al who showed that the aptamer S14 was sufficiently sensitive in an ELONA assay to detect patient samples determined as positive by RT-qPCR. In a separate study, the aptamer CoV-RBD-1C was able to detect virus-spiked

human nasal fluid samples down to virus levels of 3.67×10^2 PFU/mL in a sandwich ELONA format, showing an encouraging level of sensitivity [126]. The limit of detection of these aptamers established with purified recombinant spike protein was 54ng for S14 and 0.102ng for CoV-RBD-1C, which was similar to the performance of our SOMAmers by ELONA (40 nG to 4ng) , and gave confidence that detection levels were adequate to take forward into an LFD prototype format. Additionally, SOMAmers H6 and H8 may have utility as detection molecules in an ELONA-based clinical diagnostic assay.

Type of detection	Target	Recognition element	Dynamic range	Limit of detection	References
Direct ELONA	SARS-CoV-2 Wuhan-Hu-1 RBD	SOMAmer H6	25 µg/mL - 0.08 µg/mL	(0.08 µg/mL) 4 ng total	This study
Direct ELONA	SARS-CoV-2 Wuhan-Hu-1 RBD	SOMAmer H8	25 µg/mL - 0.08 µg/mL	(0.08 µg/mL) 4 ng total	This study
Aptamer Direct ELONA	SARS-CoV-2 spike antigen	S14 aptamer (SELEX: prefusion spike trimeric protein)	0.5 – 125 nM	2 nM (1.08 µg/mL) 54 ng	[125] (Gupta et al., 2021)
Aptamer Direct ELONA	SARS-CoV-2 S in buffer	CoV-RBD-1C	0 – 5 mg/mL	2.16 ng/mL 0.216 ng	[126] Shola David M and Kanayeva D (2022)
Aptamer Direct ELONA	SARS-CoV-2 S in human nasal fluid	CoV-RBD-1C	0 – 5 mg/mL	1.02 ng/mL 0.102 ng	[126] Shola David M and Kanayeva D (2022)

Table 4.4: Limits of detection of SARS-CoV-2 target protein in aptamer-based ELONA assays

In general, a trend in SOMAmer binding against wider sarbecovirus RBDs was observed that broadly aligned with the level of amino acid sequence conservation to Wuhan-Hu-1. Data indicated that subgroup 1 SOMAmers preferentially targeted conserved RBD features shared among closely related sarbecoviruses.

Of the sarbecoviruses tested, some clade 1a (WIV-1, SARS-CoV, RS4231), clade 1b (SARS-CoV-2, RaTG13) and clade 5 (Rc-o319) RBDs capable of using human ACE2 for host cell entry, showed binding by subgroup 1 pan-variant binding SOMAmers. Specifically, RaTG13 retained binding with most SOMAmers (8/16), a factor potentially associated with its high RBD identity to the SARS-CoV-2 Wuhan-Hu-1 strain (90%) (**Figures 4.6, 4.7 and 4.11**).

The residues involved in binding can only be inferred via binding responses and deduction, by discounting residues that remain the same when SOMAmer binding is lost and considering residues that are different between two RBDs. The binding responses of two key SOMAmers within subgroup 1 (pan-variant binders), namely H7 and H6, revealed differences in binding to two sarbecoviruses: WIV1, where binding occurred and Rc-o319 where binding was lost. These differences in binding response, along with additional observed patterns, are summarised in the phylogenetic tree below (**Figure 4.16**), with the H6 binding range highlighted in red and the H7 binding range in black. The alignment of the RBDs of Rc-0319 and WIV1 identified seventy-one positions where diversity occurs, representing potential amino acid changes responsible for the loss of binding.

Phylogenetic tree of Sarbecovirus RBDs rooted in Wuhan-Hu-1 (19A)

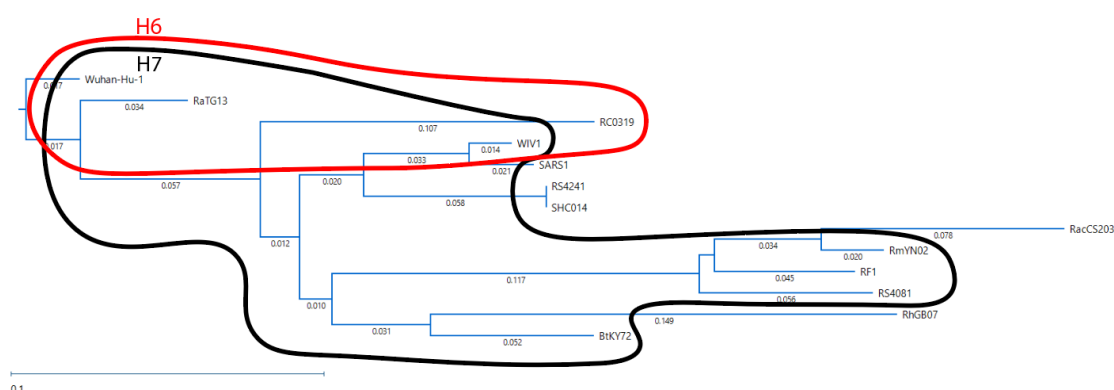


Figure 4.16: Binding ranges of H6 and H7 RBD SOMAmers on Sarbecoviruses

The red loop described the binding range of SOMAmer H6 which included RBDs from all viral species within its perimeter. Alternately, the black loop shows binding range of H7, with all species in its perimeter permissive to binding.

In contrast to the subgroup 1 SOMAmers, other subgroup 2 and 3 SOMAmers were more sensitive to sequence divergence, indicating reliance on more mutation-prone regions. Where RBD identities fell below ~70–79%, binding was restricted mainly to broadly reactive SOMAmers, such as H7, E8 or E9 (**Figure 4.11**).

The wider alpha- (PRCV, 229E, NL63), beta- (OC43, HKU1, MERS) and gamma- (IBV) CoVs full length spike proteins showed no binding to the main SOMAmer library beyond limited recognition of HKU1, although there was some binding between the Second library SOMAmers and HKU1 and 229E. This progressive loss of binding with increasing phylogenetic distance underscores the challenge of developing broadly cross-reactive affinity reagents and highlights the structural limits of SARS-CoV-2 Wuhan-Hu-1-centred SELEX selection strategies.

The aims of this chapter were to evaluate a SOMAmer library for its diagnostic potential and to down-select candidate SOMAmers ideally including 1) a wide-ranging binder and 2) a variant- or species-specific binder, for progression to epitope

evaluation. This was performed using twenty-seven recombinantly expressed RBD and FL-spike proteins across three genera of the *Coronaviridae*. This tiered approach focused on completing a binding profile of the SOMAmer library to related proteins progressing forward in evolutionary time from the emergence of SARS-CoV-2 variants through 2020–2024, and in parallel, evaluating more diverse CoVs. However, investigations showed that all SOMAmers bound multiple SARS-CoV-2 variants, and most also bound additional sarbecovirus species across all the clades (**Figure 4.11**). A minority of SOMAmers also bound a wider betacoronavirus, the Embecovirus HKU1 (**Figure 4.14**). Therefore, it was not possible to select a SOMAmer which bound a specific viral variant. Varying binding patterns of SOMAmers to a range of CoVs suggests different epitopes were used in recognition. This feature was important in identifying non-competing SOMAmer pairs for capture and detection roles in a diagnostic assay.

Chapter 5: SOMAmer epitope evaluations and development of LFD prototypes

5.1 Introduction

Following evaluation of the SOMAmer binding characteristics established in earlier chapters, this chapter focuses on translating this understanding towards a point-of-care LFT prototype. A critical requirement for LFTs is the selection of complementary, non-competing capture and detection molecules that can act as pairs, binding together on distinct epitopes present within the target analyte [108]. SOMAmers that compete for the same epitope would inhibit each other's binding, making them unsuitable for application in LFTs. Identifying SOMAmers capable of fulfilling these capture and detection roles within a sandwich ELONA format is a critical step in enabling all the elements of a point-of-care LFT to work effectively. This chapter outlines the rationale, design considerations, and experimental workflow used to develop a basic but functional ssDNA SOMAmer-based LFT format for SARS-CoV-2, that provides a foundation for future refinement and development into multiplexed or field-deployable diagnostic devices [127]. The selection of SOMAmers with differing binding patterns addresses two potential diagnostic use cases with contrasting requirements. One use case concerns pandemic preparedness, where interventions can be prepared and stockpiled in advance of a pandemic. Organisations such as CEPI and the WHO promote interventions aimed at viral families, providing the broadest base of application for rapid deployment. This approach guides SOMAmer selection toward a wide range of coronavirus binding. The second use case focuses on more specific viral detection for epidemiological studies or tailored patient diagnosis and treatment

[128]. In this scenario, SOMAmers with narrower, potentially variant-specific binding profiles should be selected.

In all cases detection elements with durable binding in the face of continuous antigenic change offer significant promise for pan-species recognition, providing greater resistance to novel variants or undiscovered viral species that may escape existing technologies [96].

5.1.1 Selection of SOMAmers for epitope evaluation and non-competitive pairing

Down-selection of ten RBD-binding SOMAmers for progression into competitive binding evaluation was determined by ELONA (**Chapter 3**) and further refined based on their binding specificity to divergent coronavirus RBDs (**Chapter 4**). Using the observed binding responses, SOMAmers could be selected for pan-variant applications, or alternatively assembled into variant-specific pairs when epitopes were known to be non-competing.

Two considerations were made when selecting SOMAmers for progression to epitope mapping. Firstly, the strength of SOMAmer binding (in this context the signal in ELONA) which is important for analyte interaction and directly impacts assay performance and the limits of detection.

Secondly, SOMAmers were selected based on the diversity of their binding patterns, as recognition of different viruses may indicate distinct epitopes being bound, thereby facilitating the identification of non-competing SOMAmer pairs. SOMAmers selected for epitope evaluation are shown in **Figure 5.1** (binding characteristics having been determined in **Chapter 4**). This includes classification into SARS-CoV-2 pan-variant or mutation-limited subgroups and assessment across a broader range of sarbecovirus RBDs.

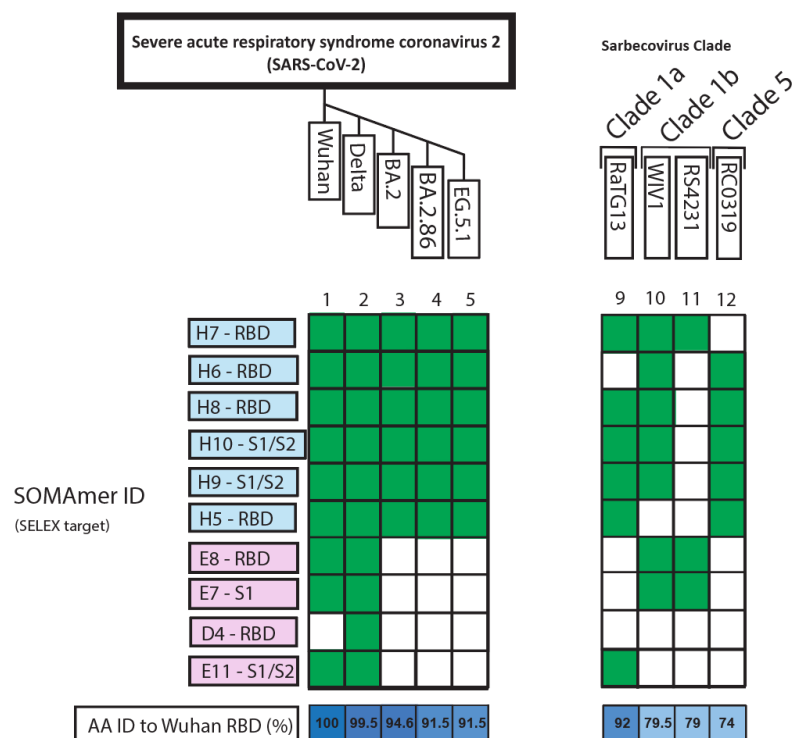


Figure 5.1: SOMAmers and viral antigens identified for epitope mapping and competitive binding analysis.

SOMAmers were chosen for strong and diverse RBDs binding responses across five SARS-CoV-2 variants and four sarbecoviruses across three clades 1a, 1b and 5. ELONA analysis is expressed as green squares indicating SOMAmer binding, with white squares showing no binding.

SOMAmers shown with a light blue background (H7, H6, H8, H10, H9, H5) are pan-variant binders, and SOMAmers shown with a pink background (E8, E7, D4, E11) are mutation-limited binders. The amino acid identity of the RBD of each virus compared to SARS-CoV-2 Wuhan-Hu-1 is shown in darker blue background.

5.1.2 Investigation and identification of non-competing SOMAmer epitopes

Two technical approaches were used to investigate competitive binding between the shortlisted SOMAmers. The first approach adapted the developed indirect ELONA to a competitive format. The second approach used a high-throughput antibody characterisation platform, the LSA system by Carterra Inc. (Label-Free Surface Plasmon Resonance Array). This had the potential to provide robust epitope

classification against multiple targets simultaneously. A further advantage of Carterra LSA SPR analysis is the ability to measure binding kinetics of SOMAmer with target proteins. Parallels can be drawn with characterising the binding kinetics of Mabs for LFT applications [129, 130]. Additional benefits of this second approach included the reduced requirement for reagents, compared with our competition-based ELONA which required substantial quantities of viral antigen and blocking reagents.

Each SOMAmer was tested individually against each of the ten SOMAmers in a series of one-to-one competition assays. To facilitate competitive binding studies our downselected SOMAmer library was provided in two forms: amine-form and biotin-form. Using the biotin-form of a SOMAmer in isolation serves as a positive control for binding to target antigens. In the competition assay format, pre-exposure of antigen to amine-form SOMAmers leads to blocking of epitopes, preventing subsequent binding of biotin-form SOMAmers. In the competition ELONA, antigen was pre-combined with a single amine-form SOMAmer allowing binding to the epitope. This mixture was then incubated with a single different biotin-form SOMAmer to allow either binding to a separate epitope or reduced binding due to epitope blocking. Subsequent incubation with HRP-streptavidin indicated the level of biotin-SOMAmer binding, which was compared with biotin-form-only control. A reduction in HRP-streptavidin binding compared with the control indicated epitope competition, and conversely the absence of reduced binding in competition wells indicates non-competing epitopes. The same concept was applied for the Carterra-LSA competition assays, where a single amine-form SOMAmer either blocked or did not compete with the interaction of a single different biotin-form SOMAmer with antigen, indicating shared or non-competing epitopes.

Target viral antigens for competitive binding studies included RBDs from a broad spectrum of SARS-CoV-2 variants, spanning evolutionary divergence from Wuhan-Hu-1 to EG.5.1. In parallel, sarbecoviruses from distinct phylogenetic clades were selected to represent viruses closely related to SARS-CoV-2 (e.g. RaTG13) [50] and those with greater diversity (e.g. Rc-o319) [131] to further examine cross-reactivity.

5.1.3 Capture and detection roles in lateral flow tests

The development of prototype lateral flow diagnostics is based on adoption of a sandwich ELISA/ELONA format (**Figure 5.2**). In this example the LFT is based on monoclonal antibodies performing capture and detection roles, which we propose to replace with SOMAmers. Ultimately, the relationship between SOMAmer molecules and whether they recognise shared or distinct epitopes on the target antigen is critical to their ability to form a stable tripartite interaction complex.

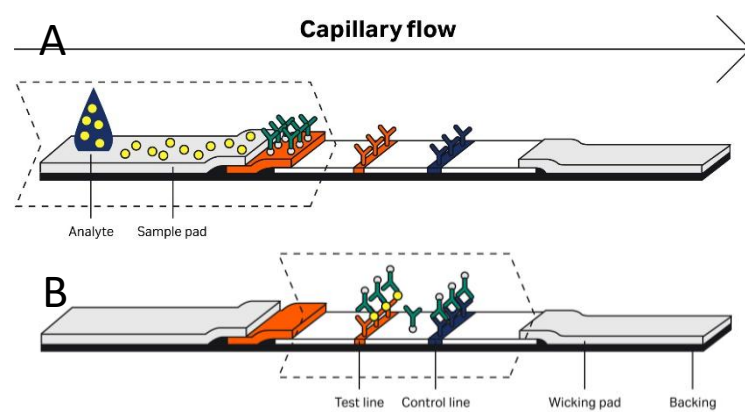


Figure 5.2: Illustration of the antibody-based lateral flow diagnostic test mechanism of action.

- A. Initial stage. Viral antigen (yellow) starts to migrate in sample buffer by capillary flow, moving along the sample pad towards the gold nanoparticle-tagged detection antibodies (green). The test line is the location of the capture agent (orange) anchored to nitrocellulose. Anti-species antibodies (dark blue) are immobilised to nitrocellulose at the control line ready to capture unbound gold nanoparticle-tagged detection antibody.
- B. End stage. As the sample and components migrate by capillary flow reaching the wicking pad, viral antigen is captured in a tripartite interaction (capture agent, viral antigen, gold nanoparticle-tagged detection agent) at the test line. Gold nanoparticles align in high density as a visible band indicating a positive test result. Unbound excess gold nanoparticle-tagged antibody continues to migrate and is captured by immobilised anti-species antibody at the control line. Gold nanoparticles align as a visible band at the control line, indicating that the test has performed as designed.

5.1.4 Stages of development of prototype lateral flow tests

The iterative process of prototype development (**Figure 5.3**) from identifying and characterising binding elements through to a validated product is undertaken in stages, typically taking 6 to 24 months to complete.

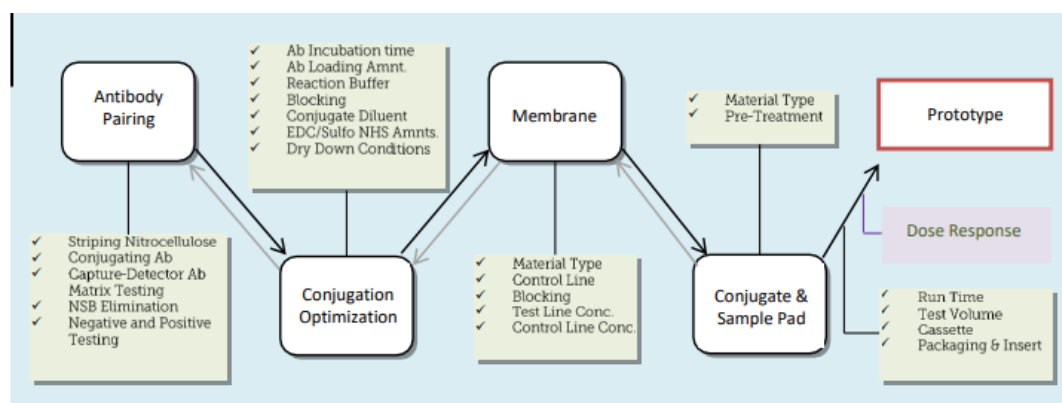


Figure 5. 3: Iterative stages in developing a prototype lateral flow test.

Commercial example derived from Stratech.

During a short industrial placement under the direction of EDX Medical, early feasibility studies were conducted in line with the early stages of development shown in **Figure 5.3**. Using the expertise and approaches of EDX Medical, the performance of key elements was assessed, including conjugation of SOMAmers to Au-NPs and evaluation of their function in binding their corresponding antigen targets. The aim was to demonstrate the components interacting successfully, and address the thesis hypothesis: can SOMAmers replace antibodies in a prototype lateral flow test?

Lateral flow diagnostics rely on the sequential interaction of three components to function effectively and produce a visual signal, as shown in **Figure 5.2**. Each of these stages depends on sufficient abundance of both the detection molecule and the target antigen, which must be determined experimentally. Additional elements such as SOMAmer dispensing volumes, concentrations of interacting components including viral antigens, and the impact of non-specific blocking competitors on binding specificity were also investigated. Other components of the LFT development

pathway, such as choice of sample membranes (typically nitrocellulose with varying flow rates and binding capacities), sample and conjugate pads (commonly cellulose, glass fibre, or polyester), and a running buffer (with defined pH, salts, detergents, and blocking reagents) to control flow, stability, and signal performance were not fully investigated in these early studies, as would be expected in a full device optimisation and validation process.

5.1.5 Justification of RBDs as a proxy for live virus or patient samples.

The recombinant RBD proteins from various sarbecovirus spikes was used as a proxy for virus during prototype development.

SARS-CoV-2 remains classified as a biosafety level 3 organism due to its infectivity and pathogenicity, which necessitates a high-containment environment. Training requirements, limited facility availability, and safety considerations would have posed significant barriers to application of live virus early in the development phase of this SOMAmer-based LFD. In addition, RBD can be expressed, purified, and quantified more readily than the full-length spike or intact viral particles. This provides greater experimental control over assay development as well as validation, facilitating the establishment of a screening assay for binding interactions.

Whilst RBD-based assays do not capture the complete context of virus–SOMAmer interactions—such as the full-length spike in its quaternary trimeric form or the contributions of other viral structural elements—the use of RBD nevertheless provides a safe, quantifiable, and diagnostically relevant antigen for prototype development prior to clinical testing. For these reasons, RBD was chosen as the early phase screening tool, representing viral diversity across the spike proteins of coronaviruses.

5.2 Results

5.2.1 Development of an ELONA-based SOMAmer competition assay.

A pilot experiment to confirm self-to-self SOMAmer binding inhibition was carried out, wherein competitor SOMAmer molecules (amine form) would occupy a single binding epitope preventing matched SOMAmers (in their biotin form) interacting with the protein target.

A reduction of binding, when compared to the biotin-form only control, should be present indicative of competition for binding to epitopes on the target antigen (**Figure 5.4**). However, in general there was no evidence of reduced binding by biotin-form SOMAmers following competition with their amine-form counterparts. Only small, potential competition effects were observed, e.g. in the interaction between biotin and amine forms of SOMAmer E11, where the competed OD450 was 1.60 compared with the non-competed biotin-only control where the OD450 was 1.98 (**Figure 5.4**). This small reduction in signal perhaps indicates that binding epitopes were not sufficiently occupied, failing to generate meaningful inhibition.

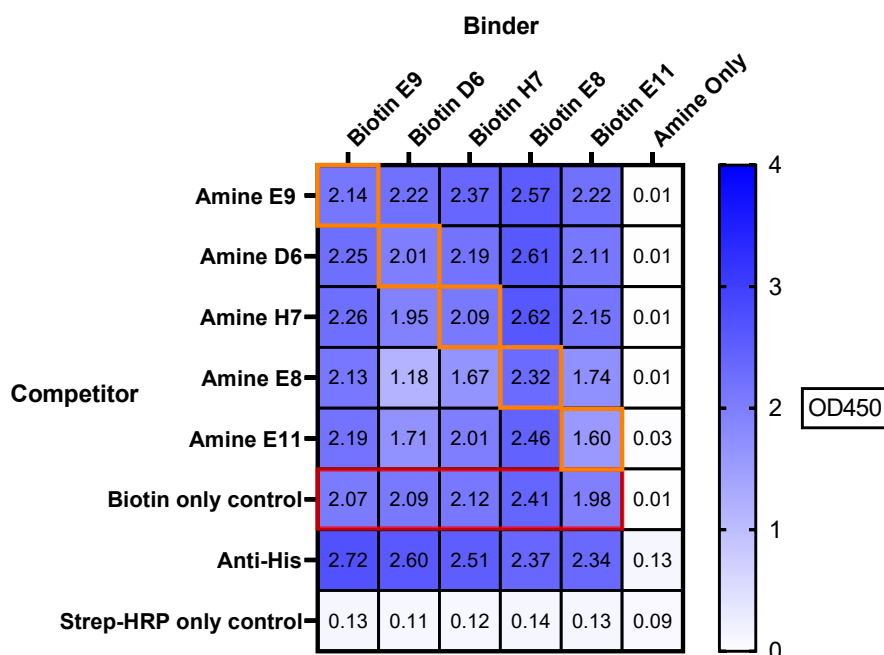


Figure 5.4: Pilot experiments investigating competition ELONA between SOMAmers in a checkerboard format.

Wells were coated with 80 ng of SARS-CoV-2 Wuhan-Hu-1 RBD, blocked with 10 μ M Z-block, washed and incubated with 30 nM unreactive amine-form SOMAmers to occupy epitopes. A matched biotin-form SOMAmer (10 nM) was then applied to wells and detected by standard ELONA. Anti-His confirmed abundance of antigen. Amine-form SOMAmer only, and Streptavidin-HRP only controls confirmed minimal background binding signal. Binding response data averaged from triplicate technical repeats.

Red outlined boxes represent uninhibited biotin SOMAmer only control for each

One factor potentially contributing to the absence of competition was a loss of binding activity, or indeed overall stability, by the amine-form SOMAmers. To investigate this, ssDNA polyacrylamide gels in 7M urea were run to assess the nucleotide size range of both the amine and biotin SOMAmers, confirming the absence of degradation, and to verify the SOMAmer stock concentration. Although information on SOMAmer sequence identity and/or length remained confidential and unavailable within the project, each SOMAmer ran approximately at the range of a 60 to 70 nucleotide ssDNA product (**Figure 5.5**). Based on the assumption that the average molecular weight of a nucleotide is 330 Daltons, SOMAmers in this project are approximately 20 kDa in size. This falls within the expected size range of ssDNA aptamers/SOMAmers [132].

Gel electrophoresis also confirmed a comparable size range between matched amine and biotinylated SOMAmers, suggesting that the absence of binding competition was not due to the amine SOMAmers being degraded.

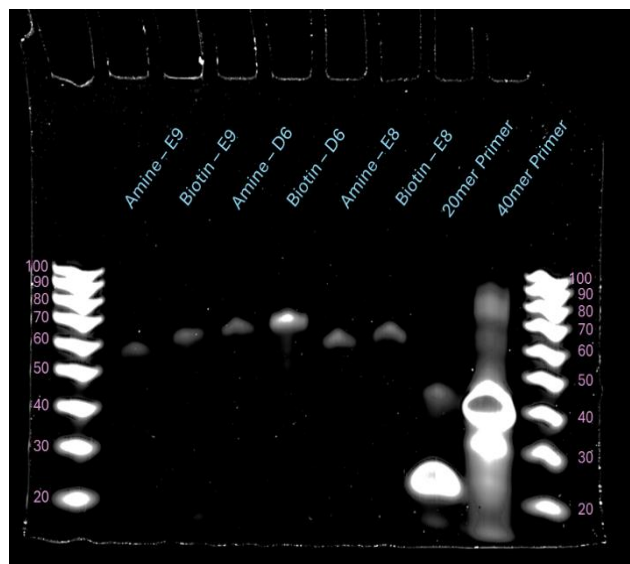


Figure 5.5: ssDNA agarose gel of SOMAmers evaluating molecular weight and abundance.

Representative SOMAmers (6.32 pmol), both amine and biotin versions, were run on a 12.5% polyacrylamide gel in the presence of 7 M urea. A ssDNA ladder of oligonucleotides ranging between 20 and 100 nt lengths, and primers with sequence lengths of 20 and 40 nt were run in parallel to SOMAmers as molecular weight comparators. ssDNA was visualised with Sybr Gold.

Further steps towards optimisation of the competition assay included (a) altering the ratio of competitor amine-form SOMAmer to biotin-form SOMAmer and (b) altering the abundance of coated viral RBD present in the assay interactions. If the target antigen is always present in excess, both the competitor and the binder SOMAmer can interact with the antigen independently, limiting competition and preventing observable inhibition. Focusing on one SOMAmer, the ratio of amine-form SOMAmer H7 to its biotinylated-form was altered through a titration range of 8:1 amine-biotin down to 0.625:1. The 8:1 amine to biotin ratio showed a marked decrease in biotin signal compared with the biotin only control, indicating effective competition (**Figure 5.6A and 5.6B**) for the same RBD epitope. In a second experiment, the concentration of

viral RBD antigen used for coating was reduced from 1.6 $\mu\text{g/mL}$ (**Figure 5.6A**) to 0.4 $\mu\text{g/mL}$ (**Figure 5.6B**). The pattern of competitive binding by the SOMAmer pairs at each amine to biotin ratio was comparable at both RBD antigen concentrations, indicating that this was unlikely to be a major contributing factor in preventing competition.

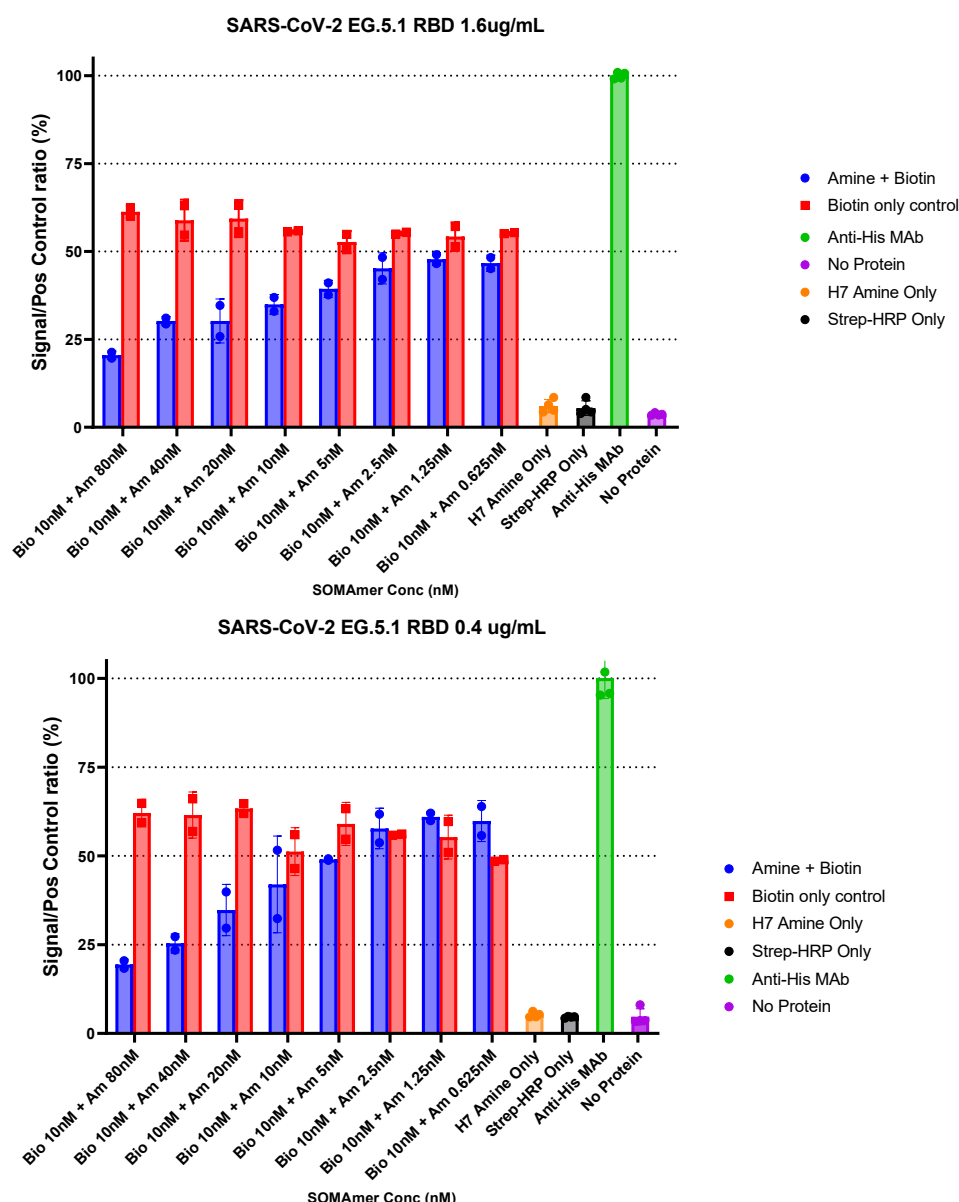


Figure 5.6: Titration of amine SOMAMers in indirect competition ELONAs.

- Ratio of H7 amine-biotin titration at 1:8 ranging to 1:0.0625 against 80 ng (1.6 $\mu\text{g/mL}$) of SARS-CoV-2 RBD of EG.5.1 variant. Signal expressed as ratio of anti-His positive control to normalise data across experiments.
- Ratio of H7 amine-biotin titration at 1:8 ranging to 1:0.0625 however each well coated with 20 ng (0.4 $\mu\text{g/mL}$) of SARS-CoV-2 RBD of EG.5.1 variant. Signal expressed as ratio of anti-His positive control to normalise data across experiments.

Binding response data averaged from triplicate technical repeats.
experimental details identical as in Figure 5.4.

Although using higher ratios of amine to biotin SOMAmer in these competition assays showed self-competition, the approach required high quantities of reagents and was relatively low throughput. This approach was therefore not used further for evaluation and mapping of competition groups between multiple SOMAmers, and an alternative technology, adopting high throughput SPR analysis was adopted.

5.2.2 SOMAmer competition assays: identification of shared epitopes by Carterra LSA SPR analysis.

Surface plasmon resonance (SPR) offers a label-free format for evaluating interactions between molecules and target analytes used in vaccine, therapeutic, and diagnostic antibody development. This technique correlates changes in the refractive index at sensor surfaces with binding events as antigens interact with immobilised capture reagents. These interactions, monitored in real time, can be used to determine epitope groupings through a checkerboard of binding competitions. In my context, each SOMAmer was assessed for competition with itself and with other SOMAmers to determine whether they recognise the same or overlapping epitopes. The Carterra LSA's advantage over standard SPR lies in its significantly higher throughput and in the evaluation of SOMAmers in both orientations (e.g., H7–Biotin vs. H8–Amine; and H7–Amine vs. H8–Biotin). Interactions were performed twice, once with SOMAmer A immobilised and SOMAmer B injected, and again with SOMAmer B immobilised and SOMAmer A injected. This bi-directional testing reduces false interpretations and provides an assessment of competing and non-competing epitopes.

The conclusions drawn from these experiments informed SOMAmer pairing strategies and performance characteristics, feeding into my later diagnostic assay optimisation and prototype development. This analysis also provided insight into how epitope groups shift with antigenic diversity and mutation, from SARS-CoV-2 variants to more divergent sarbecoviruses.

Of note, the health and performance of streptavidin-coated Carterra LSA SPR chips was monitored throughout the epitope-mapping experiments to assess loading capacity following buffer clearance of immobilised SOMAmers and chip surface regeneration. The cutoff for determining binding or blocking responses was defined using predetermined threshold values. During SPR analysis, each SOMAmer was classified into an epitope bin, defined as individual SOMAmers that compete with each other and therefore likely bound to the same or overlapping epitope.

A checkerboard format of binding competitions between SOMAmers was applied to each viral antigen. An example of the data produced for competitive binding to SARS-CoV-2 Wuhan-Hu-1 RBD is shown in **Figure 5.7A**, where a red box indicated interactions were blocked by competition between SOMAmers, and a green box indicates that both SOMAmers could bind to the target antigen, indicating separate epitopes and therefore no competition. The checkerboard interaction data is alternatively displayed as network plots for SARS-CoV-2 Wuhan-Hu-1 (**Figure 5.7B**) where competing SOMAmers are grouped together into 3 networks for this target antigen, showing blocking interactions and distinguishing separate epitope groupings. The subsequent network plots for SARS-CoV-2 variants are displayed in **Figure 5.7C**, and for sarbecoviruses in **Figure 5.7D**. For each viral RBD investigated, SOMAmers H5, H6, H8, H9 and H10 fell into a single network group indicating a common epitope. A second SOMAmer library group of H7, E7 and E8 appeared to bind to a separate epitope for SARS-CoV-2 Wuhan-Hu-1, Delta, RaTG13 and WIV1 RBDs. These two groups therefore provided non-competing SOMAmers for capture and detection pairings.

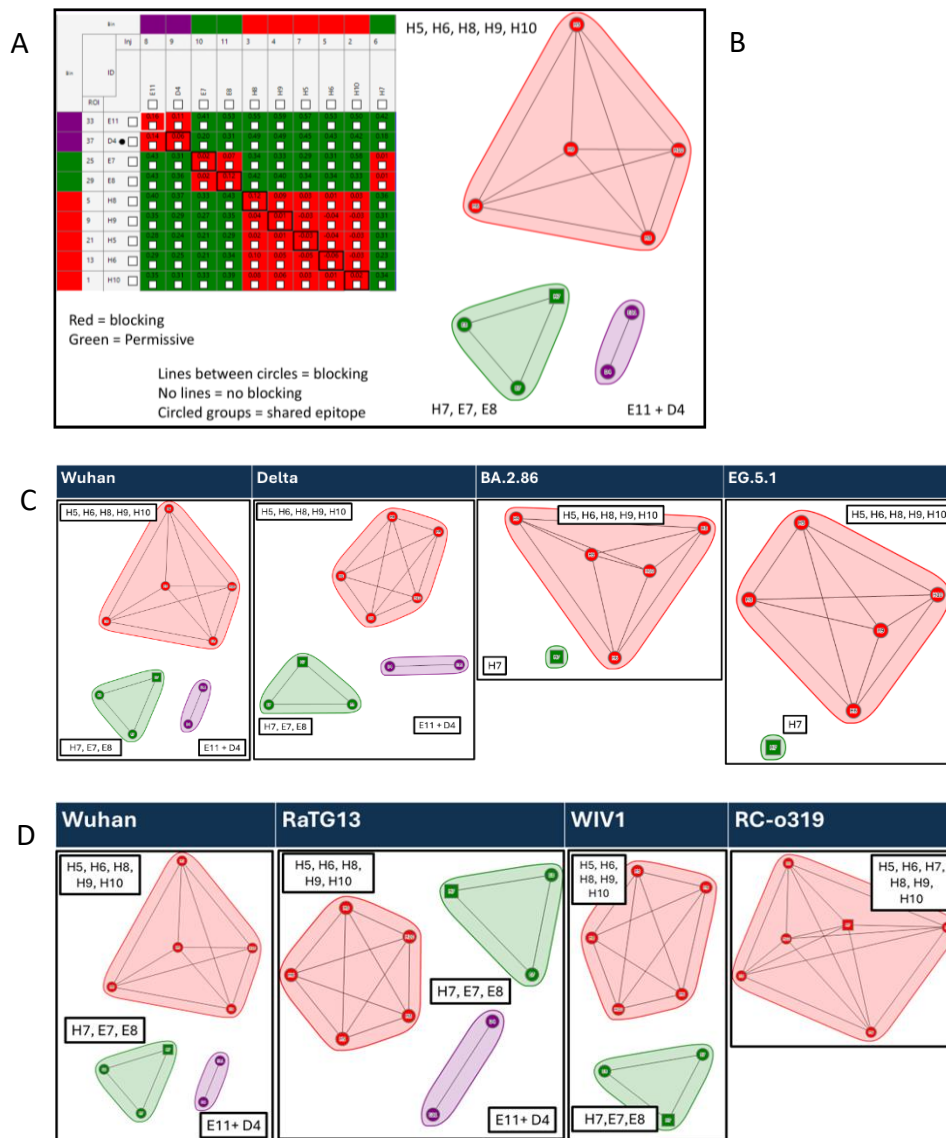


Figure 5. 7: SARS-CoV-2 Wuhan-Hu-1 SOMAmers epitope groups determined by Carterra LSA SPR.

A. Competition checkerboard illustration of SOMAmer interactions for Wuhan-Hu-1 RBD (x-axis and y-axis shows each SOMAmer competed with itself and each other SOMAmer), also applied to other viral RBDs.

B. Binding competition data displayed as network plots sorted into three groups of SOMAmers sharing a common epitope (red H5, H6, H8, H9, H10; green H7, E7, E8; and purple E11, D4) for Wuhan-Hu-1 RBD.

C. Binding competition data displayed as network plots sorted into groups of SOMAmers sharing a common epitope (red, green and purple) for SARS-CoV-2 variants (Wuhan-Hu-1, Delta, BA.2.86 and EG.5.1).

D - Binding competition data displayed as network plots sorted into groups of SOMAmers sharing a common epitope (red, green and purple) for sarbecoviruses (RaTG13, WIV1 and Rco-319).

Binding response data averaged from triplicate technical repeats, with both Antigen and SOMAmer immobilized to chip surface.

In addition to the range of binding, the kinetics of these interactions are equally critical, such as the rate of complex formation with the target, referred to as the association rate (k_a), followed by the rate at which complexes dissociate (k_d).

In a diagnostic format, favourable kinetics are essential for achieving a high abundance of tripartite complexes, which directly results in greater accumulation of gold nanoparticles immobilised at a specific location in the prototype LFT. Higher abundance leads to increased colour intensity, producing a darker visual signal, which is a key factor for achieving adequate sensitivity in the absence of signal amplification. The kinetic performance of each SOMAmer across different RBDs representing SARS-CoV-2 variants and sarbecoviruses may also vary, providing insight into the wider viability of specific SOMAmer pairs for detecting divergent coronaviruses simultaneously in an LFT format. For example, a low SOMAmer association rate and high dissociation rate toward one specific viral antigen could reduce test performance.

5.2.2.1 - K_a (association rate)

Association (k_a) describes the rate at which a ligand binds to its target and is measured in moles per second ($M^{-1}s^{-1}$). K_a works together with k_d to define the overall binding affinity ($KD = k_d/k_a$). A high association rate indicates that a SOMAmer or monoclonal antibody could excel not only in therapeutic applications but, more importantly in diagnostic contexts.

Lateral flow tests operate under non-equilibrium conditions, where antigens moving within buffer interact only briefly with capture elements before being drawn toward the wicking pad. In the presence of low-abundance antigen, such as during early infection, a high k_a increases the probability of binding and accelerates signal generation, both of which are critical for point-of-care testing.

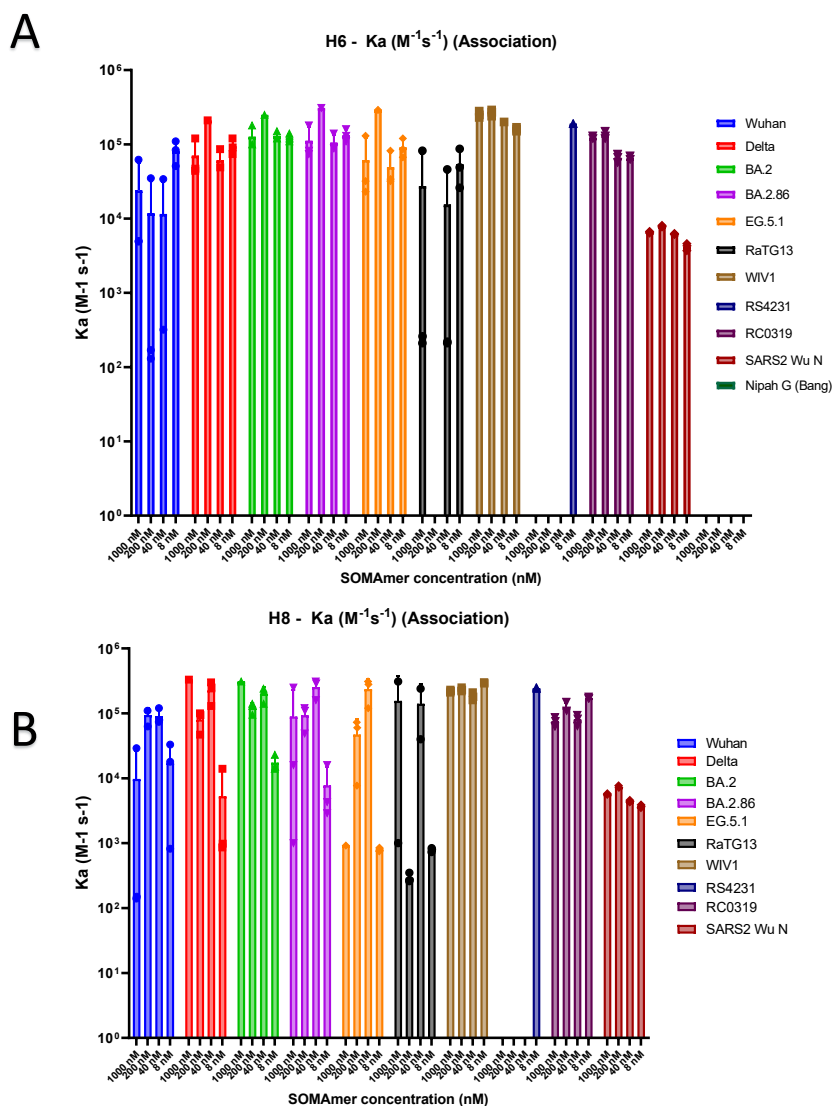


Figure 5.8:
Association kinetics of specific SOMAmers against a range of sarbecovirus RBD targets, as determined by SPR.

A. SOMAmer H6

B. SOMAmer H8

A range of concentrations of selected SOMAmers (1000 to 8 nM) were analysed by Carterra LSA SPR for association kinetics against a range of sarbecovirus RBD targets. SARS-CoV-2 nucleocapsid antigen was included as a control. Binding response data averaged from triplicate technical repeats.

The kinetic analysis of all ten SOMAmers was examined by SPR, and selected data are shown here. The k_a association rates of SOMAmers H6 and H8 binding individually to the recombinant RBDs from various coronaviruses were investigated by SPR. For some SOMAmer:RBD interactions, repeated association rate measurements at varying concentrations of SOMAmer gave consistent results as can be seen in the bars representing H6 binding to Delta, BA2, and BA.2.86 RBDs (**Figure 5.8A**). However, there were more variable results for other SOMAmer:RBD binding reactions with varying SOMAmer concentrations (**Figure 5.8A and B**: H6 binding to Wuhan RBD; H8 binding to Delta, BA.2, BA.2.86, EG5.1 and RaTG13 RBDs), with some

interactions not giving a readout at all (**Figure 5.8A**: H6 against RaTG13 RBD). This inconsistency is not understood but is thought to be likely due to technical reasons. Unfortunately, SOMAmer H7 interactions were unable to withstand the SPR regeneration buffer cycles during the kinetic analysis, so no data was available. For all sarbecovirus RBDs tested, the association kinetics of SOMAmer H6 fell within the typical range of mAb binding characteristics [129, 130, 133] of 10^4 to 10^6 $M^{-1} S^{-1}$ (**Figure 5.8A**). In comparison, SOMAmer H6 showed a slower association speed of 10^3 to 10^4 $M^{-1} S^{-1}$ with the negative control protein SARS-CoV-2 nucleocapsid as its binding target, as expected.

The association kinetics of SOMAmer H8 were more variable (**Figure 5.8B**) with typical mAb binding characteristics of 10^4 to 10^6 $M^{-1} S^{-1}$ for some sarbecovirus RBDs tested (SARS-CoV-2 variants Wuhan-Hu-1, Delta, BA.2, BA.2.86; and wider sarbecoviruses WIV1 and RC0319). However, H8 showed a slower association speed of 10^3 to 10^4 $M^{-1} S^{-1}$ with SARS-CoV-2 variant EG.5.1 and RaTG13.

5.2.2.2 - *K_d* (dissociation rate)

The second aspect of molecular kinetics is dissociation, or the speed at which ligand–target complexes break apart. Measured in s^{-1} , k_d describes the performance of molecular recognition elements after complexes have formed.

The dissociation rate influences design choices and optimisation, as this contributes directly to a test's analytical sensitivity and limit of detection. In lateral flow tests, which operate as desiccated sandwich ELISAs, continued binding and a slow dissociation rate of the captured antigen are essential for proper test function. If complexes do not remain bound, the detection and capture molecules cannot engage for a sufficient duration to generate a visible spot or line.

K_d contributes directly to overall affinity through the relationship $KD = k_d / k_a$. Lower k_d values represent slower dissociation and higher binding stability, whereas higher k_d values reflect faster dissociation and lower stability. For example, a k_d of $1 \times 10^{-5} \text{ s}^{-1}$ represents the dissociation of only 0.001% of complexes each second, indicating a highly stable interaction.

The dissociation rates of SOMAmers H6 and H8 at varying concentrations were evaluated against recombinant RBDs from various coronaviruses by SPR as displayed in **Figure 5.9**. SOMAmer binding produced variable results across concentrations. The method of evaluating SOMAmers with the Catterra SPR system resulted in technical challenges specifically with SOMAmer H7 interactions which were unable to withstand the SPR regeneration buffer cycles, resulting in no usable data.

The dissociation kinetics of SOMAmers H6 and H8 fell within the typical range of monoclonal antibody binding characteristics. The performance of these SOMAmers was sustained across SARS-CoV-2 variants and the wider sarbecoviruses tested, excluding BA.2.86, EG.5.1 and RaTG13.

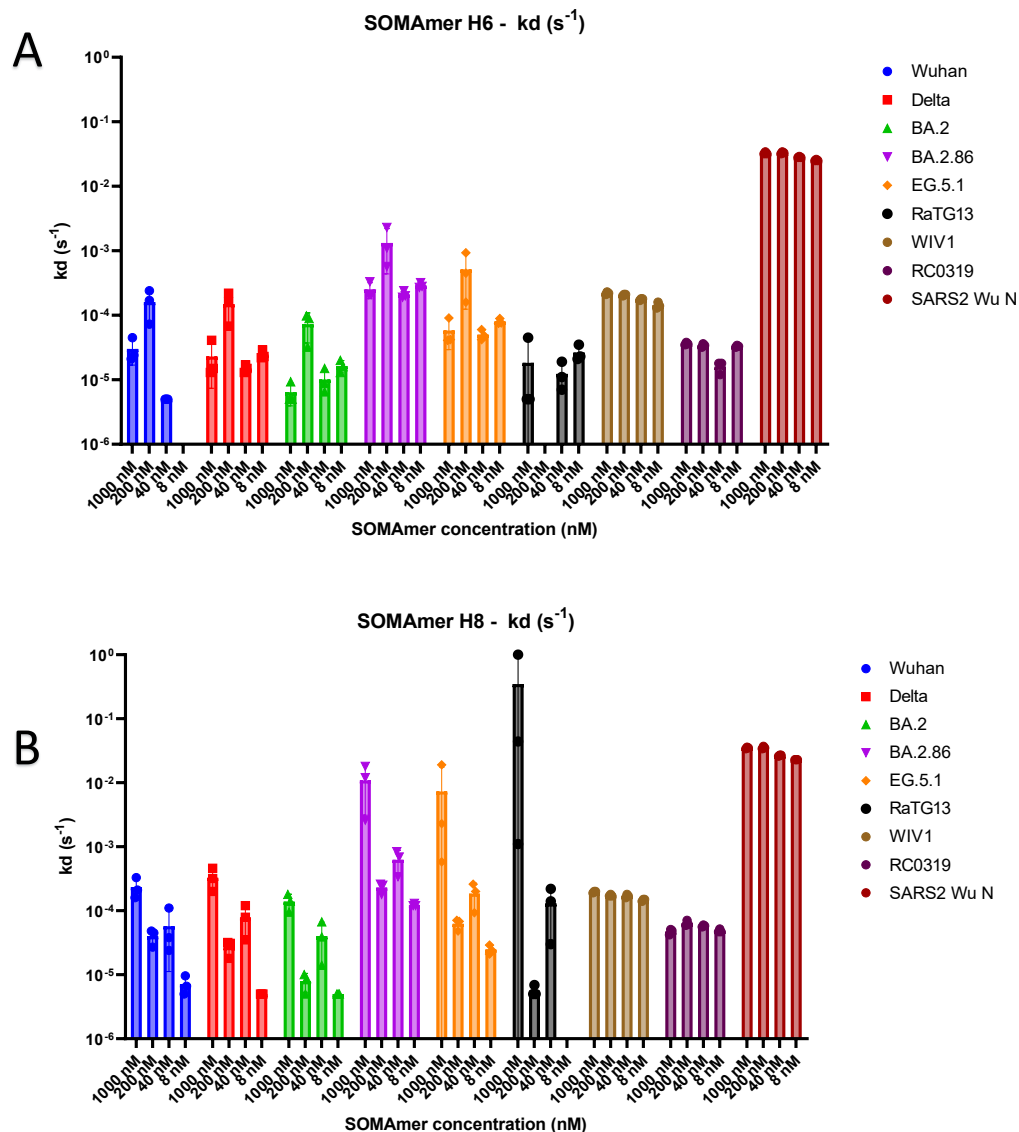


Figure 5.9: Dissociation kinetics of selected SOMAmers with a range of sarbecovirus RBDs, as determined by SPR.

A. SOMAmer H6

B. SOMAmer H8

A range of concentrations of specific SOMAmers (1000 to 8 nM) were analysed by Carterra LSA SPR for dissociation kinetics, against a range of sarbecovirus RBD targets. SARS-CoV-2 nucleocapsid antigen was included as a control. Binding response data averaged from triplicate technical repeats.

5.2.2.3 - *KD* (Binding equilibrium)

The balance between association and dissociation is represented by binding equilibrium, defined as the concentration at which 50% of the available target is bound and reflecting the balance between the previously mentioned association and dissociation rates. This measurement forms the basis for describing binding strength. For SOMAmers, comparing their K_D values to those of antibodies indicates how valuable they may be as potential replacements in diagnostics such as LFTs. Lower K_D values indicate tighter binding affinities, whereas higher K_D values reflect weaker interactions. Despite rapid LFT diagnostic assays not operating under equilibrium conditions, measuring K_D s against various representative SARS-CoV-2 variants and sarbecovirus RBD provides insight into the kinetic performance of each SOMAmer. The equilibrium constant of each SOMAmer was calculated using the measured k_a and k_d against the various RBDs tested. As previously mentioned, SOMAmer H7 interactions were unable to withstand the SPR regeneration buffer cycles, resulting in no usable data. The equilibrium constant of SOMAmer H6 fell within the typical range of high affinity monoclonal antibody binding characteristics, as shown in **Figure 5.10** [129, 130]. The performance of this SOMAmer was sustained across SARS-CoV-2 variants and the wider sarbecoviruses tested, indicating suitability for inclusion in a diagnostic device. SOMAmer H8 showed a mixed result, with high equilibrium affinity for some viral strains (Wuhan-Hu-1, Delta, BA.2 and RCo319) but low affinity for other viral strains (BA.2.86, EG.5.1 and RaTG13).

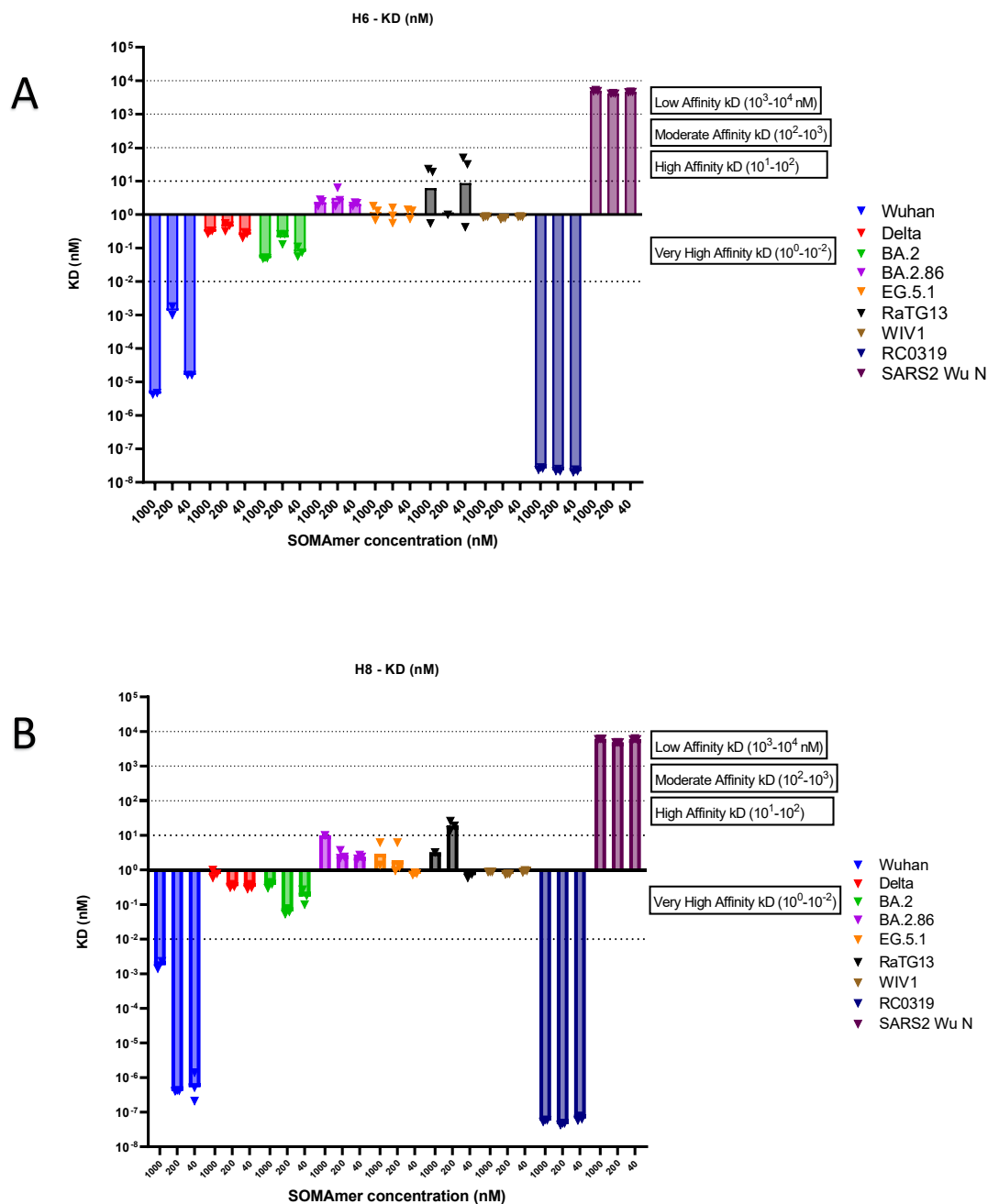


Figure 5.10: KD of specific SOMAmer candidates against a range of sarbecovirus RBD proteins.

A. SOMAmer H8, B. SOMAmer H6

B. A range of concentrations of SOMAMers (1000 to 8 nM) were analysed by Carterra LSA SPR for equilibrium constant kinetics with a range of sarbecovirus RBD targets. SARS-CoV-2 nucleocapsid antigen was included as a control. Published KD ranges of monoclonal antibodies (Pedreira and Rincon, Starr, Jensen, and Guest) and aptamers (Yang) are shown for comparison. SARS-CoV-2 nucleocapsid antigen was included as a control. Binding response data averaged from triplicate technical repeats.

The performance of each SOMAmer is comparable to equilibrium constants (KD) for monoclonal antibodies in general [12][134] [135](**Figure 5.11**), applications in LFDs [13] and pan-coronavirus neutralising antibodies [14]. Aptamer binding to SARS-CoV-2 spike regions showed KD values at low nanomolar levels [15], whereas H6 and H8 displayed binding KD values of a higher affinity. These publications express a range of responses, moving from high affinity performers to still adequate lower affinity KD values suitable for diagnostics.

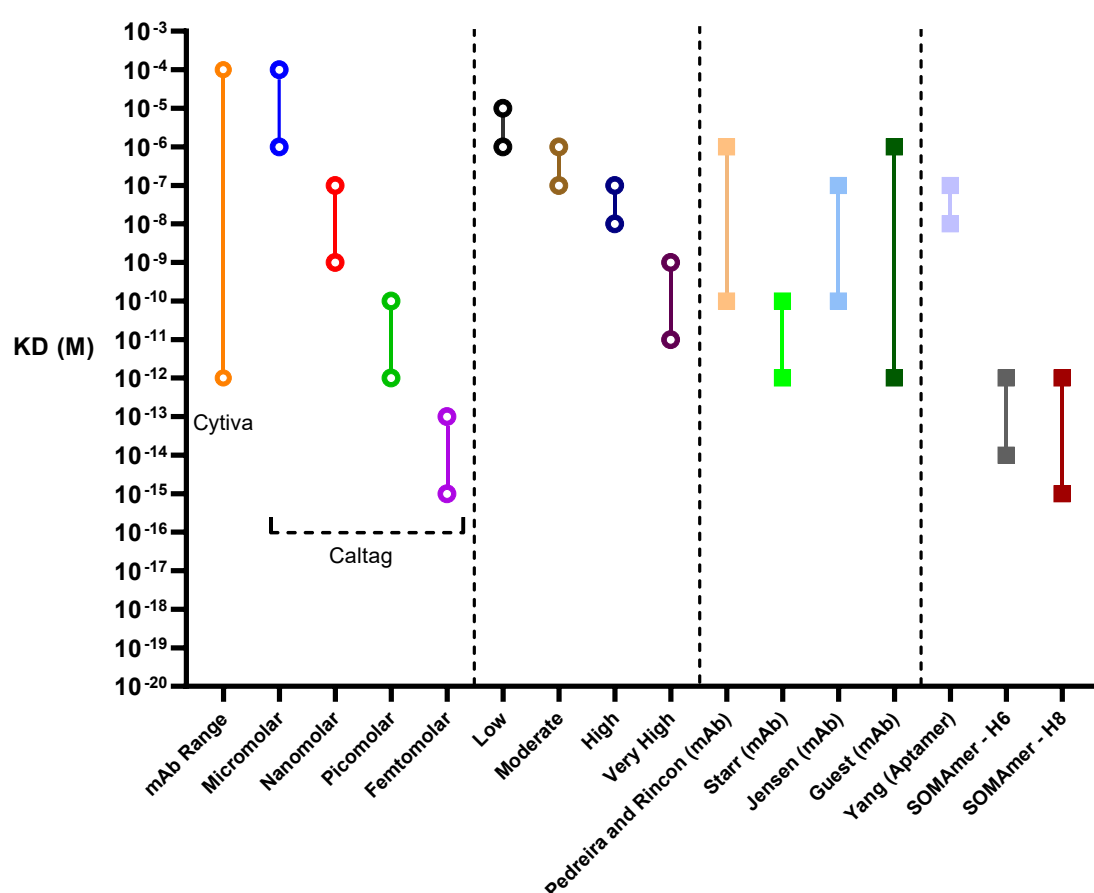


Figure 5.11: Comparison of KD values for monoclonal antibodies, aptamers and SOMAmers

KD represents the binding equilibrium, defined as the concentration at which 50% of the available target is bound, reflecting the balance between the association and dissociation rates. Lower KD values indicate tighter binding affinities, whereas higher KD values reflect weaker interactions. Data derived from multiple published sources, including Caltag Medical [134], Cytiva [135], Pedreira and Rincon [136], Starr [96], Guest [30], Jensen [137] Yang [138] and were compared with SOMAmer data for H6 and H8. Binding response data averaged from triplicate technical repeats.

5.2.3 Choice of SOMAmer pairs for prototype lateral flow test development

Following the SOMAmer competition and kinetic analysis, a small number of SOMAmer pairs were selected to take forward into prototype lateral flow development, which was to be optimised initially against the SARS-CoV-2 Wuhan-Hu-1 RBD antigen. The non-competing SOMAmer pair of H7:H6 was selected based on broad CoV binding range, the strongest ELONA signal responses, and compatible non-competing SOMAmer epitope sites. These elements combined provided the best ability to overcome the expected challenges of low visual signal strength during prototype development, when compared to other SOMAmer candidates.

Two non-specific binding control SOMAmer pairs were selected based on the combination of H7:C11 and H8:C11, as the C11 SOMAmer generated in SELEX against SARS-CoV-2 nucleocapsid protein. The SOMAmer pairs and their potential diagnostic specificity ranges are shown in **Table 5.1**.

Role	Detection SOMAmer	Specificity	Capture SOMAmer	Specificity	Expected detection
Non specific control	H7	All variants, sarbeco 8/12	C11	Binds nucleocapsid, no binding to spike or RBD	None
Test	H7	All variants, sarbeco 8/12	H6	All variants , WIV1, RCoV319, RS4231, BtKY72	All variants , clade 1b, 3, 5

Table 5.2: Prototype LFD designs based on identified SOMAmer pairs, design number, configuration of components and expected virus antigen detection range.

H7 detects the RBDs of all SARS-COV-2 variants and sarbecoviruses, as shown.

C11 detects the SARS-COV-2 nucleocapsid and is used as a negative control.

5.2.4 Generation of a prototype SOMAmer based lateral flow test (LFT) using SARS-CoV-2 Wuhan-Hu-1 RBD.

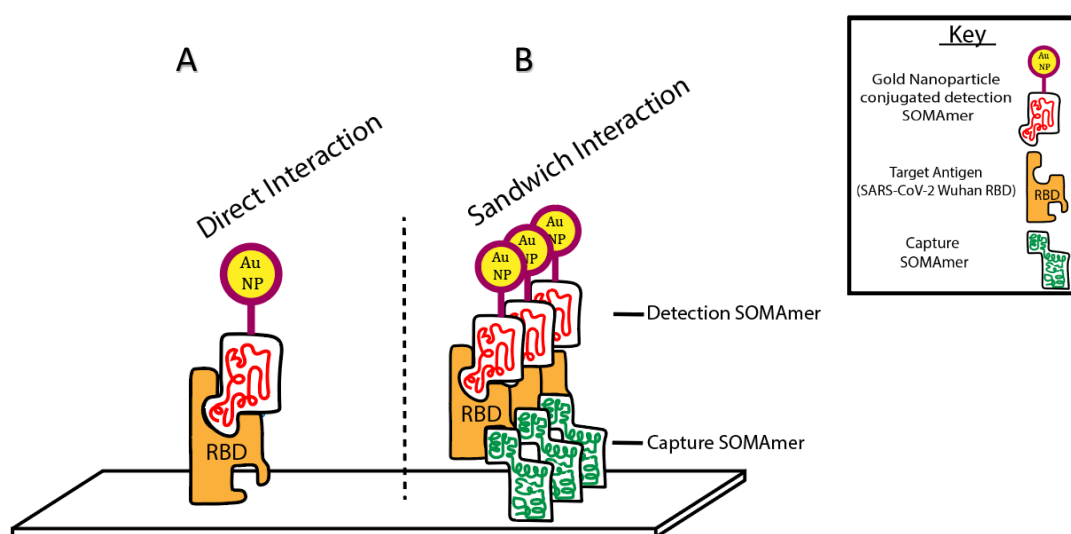


Figure 5.12: SOMAmer: antigen interactions in development and final stages of prototype LFT.

- A. Illustration of direct interaction of Au-NP conjugate detection SOMAmer binding to immobilised target antigen, SARS-CoV-2 RBD.
- B. Illustration of tripartite sandwich interaction between of Au-NP conjugate detection SOMAmer: RBD: capture SOMAmer.

5.2.4.1 Overview of prototype development steps

The following development steps were investigated in preparing the LFT prototype, which ultimately requires tripartite binding interactions as shown in **Figure 5.12B**. Steps are described in more detail below.

Firstly, the ability of Au nanoparticle (Au-NP)-conjugated detection SOMAmers H7 and H8 to bind to immobilised target antigen RBD by direct interaction was investigated in a non-LFT format (**Figure 5.12A**). This step also demonstrated that Au-NP conjugation to detection SOMAmers had been successful.

Secondly, and a step closer to the LFT format, the next stage was to apply capture SOMAmers H6 (and C11 as control) to nitrocellulose and subsequently run a pre-combined mixture of target antigen RBD and detection SOMAmer H7 and H8 by

capillary action across the capture SOMAmers. This early stage used a pipette to deposit capture SOMAmers onto the nitrocellulose surface before drying.

The next stages in development towards an LFT format was to use the Biodot™ machine to apply regular 6 nL depositions of capture SOMAmers, rather than using a pipette. The application of Au-NP conjugated detection SOMAmers to a conjugate pad, target antigen RBD concentration, and Z-block buffer concentration were investigated.

5.2.4.2 – Binding of Au-NP conjugated detection SOMAmer to immobilised RBD.

The first experiment was to investigate whether the Au-NP conjugated detection SOMAmers H7 and H8 could bind to target antigen RBD immobilised onto a nitrocellulose membrane. Two nitrocellulose strips with replicate large dots are shown in **Figure 5.13B**. The immobilised target RBD antigen was dried onto the nitrocellulose strip, and subsequently Au-NP conjugated detection SOMAmer was migrated across the antigen and captured in situ. The resulting Au-NP accumulated material was visible by eye as large dots. Both Au-NP conjugated detection SOMAmers H7 and H8 were immobilised by target antigen indicating that binding could occur successfully when components were migrating by capillary action (**Figure 5.13B**). Compared with SOMAmer-antigen complex formation in a plastic well (as used in ELONA), these binding conditions were a good initial step towards an LFT prototype. Although there was no SOMAmer capture element in this experiment, and the target antigen (sample) would not be immobilised in a final LFT, this early development step was important in establishing detection SOMAmer performance. This experiment also demonstrated the successful conjugation of Au-NP to the SOMAmers, and the integrity of the RBD target protein and its epitopes on the membrane.

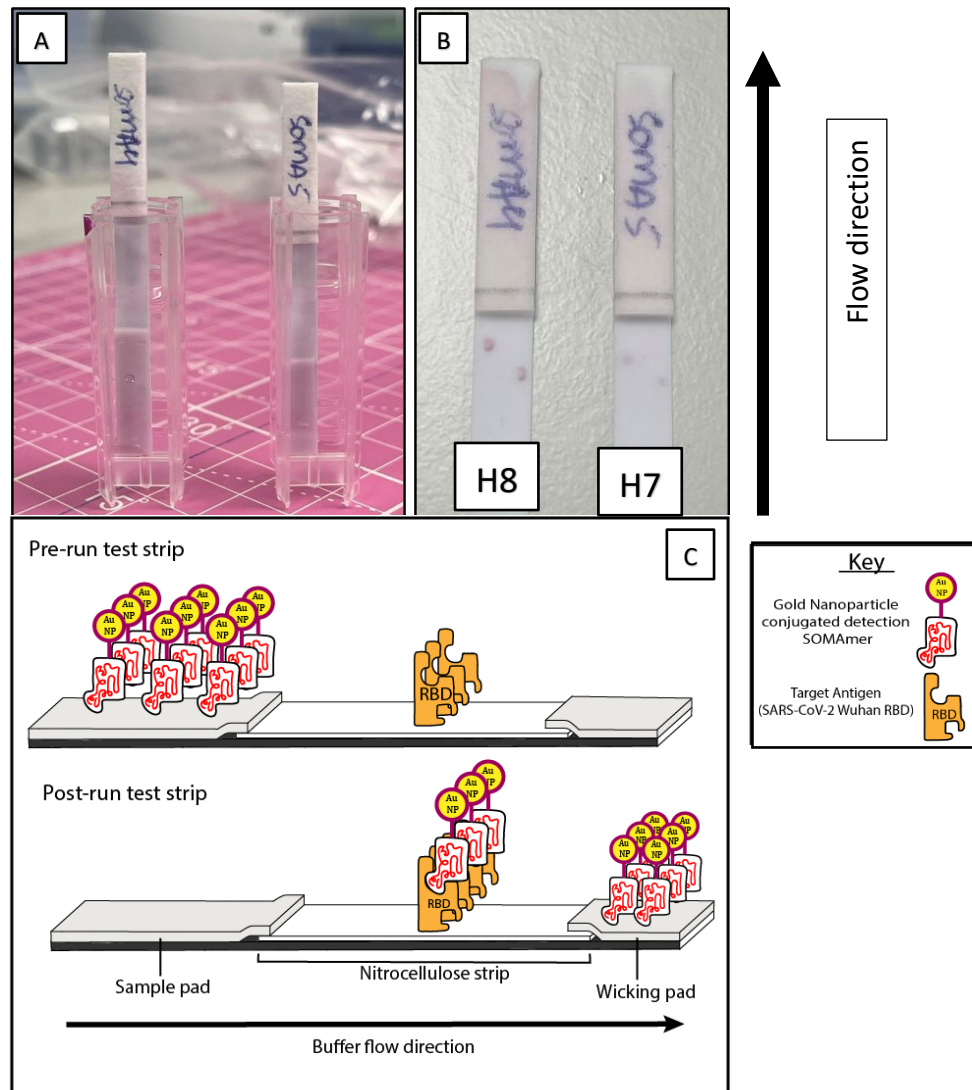


Figure 5.13: Direct interaction between a desiccated RBD antigen on a nitrocellulose surface and Au-NP-conjugated H7 and H8 SOMAMers.

- Cuvette with the end of the nitrocellulose strip immersed in buffer.
- RBD (2 ng) was deposited by pipette onto nitrocellulose strips and allowed to dry. Au-NP detection SOMAMers H7 or H8 were then prepared at a final OD of 8 in 150 μ L running buffer (100 μ M Z-block, 4.5%BSA, 1 mM MgCl_2 in PBS). Nitrocellulose strips were placed into cuvettes and buffer allowed to migrate up by capillary action. Strips were read after 10 minutes.
- Illustration of pre- and post-run test components and binding structures. Evaluations performed on single replicate.

5.2.4.3 – Binding of pre-mixed RBD and Au-NP detection SOMAMers to a capture SOMAmer previously dried onto nitrocellulose.

Having demonstrated that Au-NP conjugated detection SOMAMers were able to migrate across a nitrocellulose strip and bind target antigen, the next step was to

investigate the performance of capture SOMAmers as part of a functional pair of non-competing SOMAmers.

The capture SOMAmers H6 (and C11 as control) were first applied by pipette deposition onto nitrocellulose and then allowed to dry. Subsequently, a pre-combined target antigen RBD and Au-NP-conjugated detection SOMAmer (H7) were applied to the nitrocellulose to migrate by capillary action across the capture SOMAmers. Duplicate dots were visible relating to capture SOMAmer H6, but not to control capture SOMAmer C11(which binds to SARS-CoV-2 nucleocapsid) (**Figure 5.14**). This indicated that pre-immobilised capture SOMAmer could bind and immobilise pre-incubated RBD-detection SOMAmer complexes. In addition, my experiments demonstrated that this binding was specific for SOMAmer pairs which are directed against the SARS-CoV-2 RBD, as the control capture SOMAmer C11 (directed against SARS-CoV-2 nucleocapsid) showed no binding.

Of note, residual, excess Au-NP-conjugated detection SOMAmer was retained in the sample pad and was clearly visible as a dark patch at the proximal end of the test strips. This, however, did not affect the formation of the tripartite interaction. Nevertheless, future optimisation experiments should not overload the test strips with reagent. The overabundance of antigen and detection SOMAmer may have exceeded the capillary action of the buffer front across the test strip towards the wicking pad. Again, this demonstration that binding between dried capture SOMAmers, RBD and detection SOMAmers could occur successfully when components were migrating by capillary action was a step towards the full LFT prototype format.

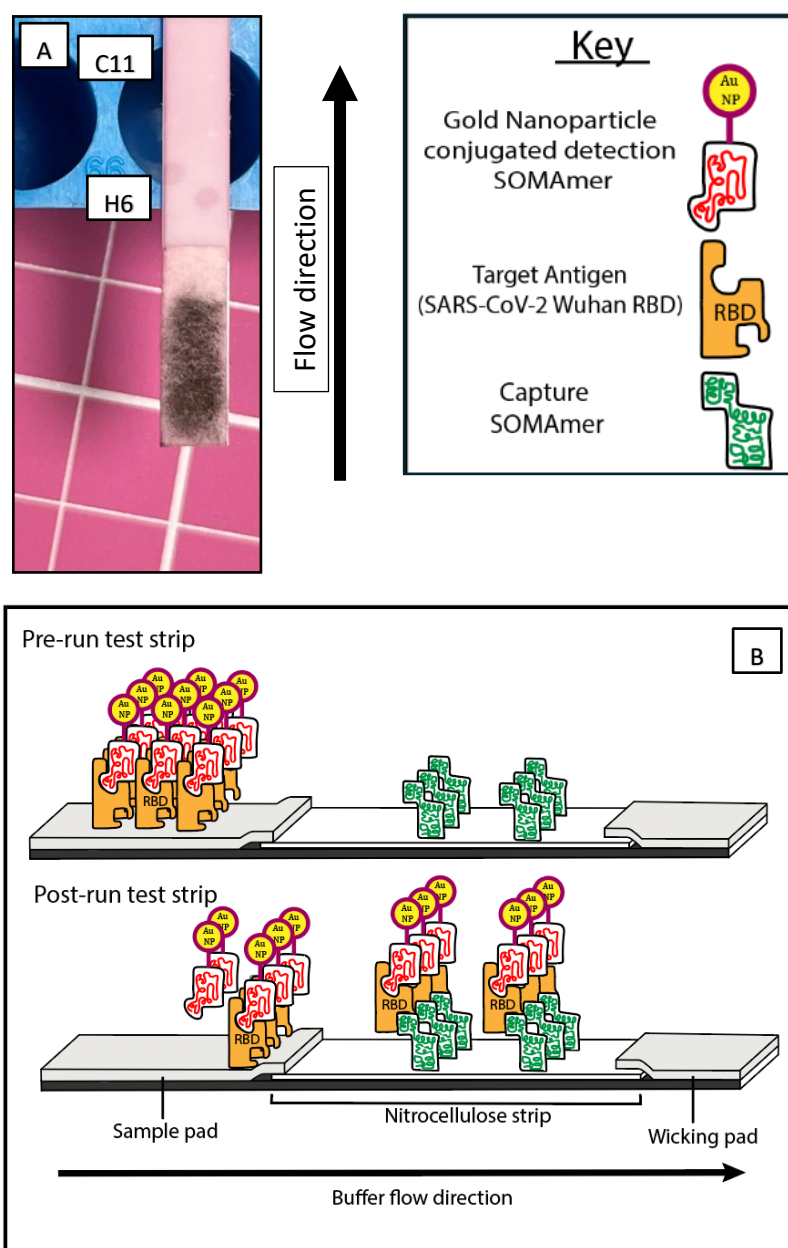


Figure 5.14:
Tripartite binding of pre-mixed RBD and Au-NP conjugated detection SOMAers with capture SOMAers previously dried onto nitrocellulose.

A. A 750 μL droplet containing 100 ng of capture SOMAmer (H6, or C11as control) was pipetted onto nitrocellulose strips and allowed to dry. AuNP-detection SOMAmer H7 was prepared at a final OD of 2 in 150 μL running buffer (100 μM Z-block, 4.5% BSA, 1 mM MgCl_2 in PBS) and pre-combined with 1.5 ng of SARS-CoV-2 Wuhan-Hu-1 RBD for 5 minutes. Pre-combined complexes were pipetted on to the sample pad and allowed to migrate across the nitrocellulose strip towards the wicking pad. Strips were read after 10 minutes. Evaluations performed on single replicate.

B. Illustration of pre- and post-run test components and binding structures.

5.2.4.4 - Biodot deposition of SOMAers onto capture roles in an LFT format

The next stages in development towards an LFT format was to use a Biodot machine to apply regular 6 nL depositions of capture SOMAers to the membrane, rather than to use pipette deposition. This technology obviously allows application of much smaller volumes of capture SOMAmer compared with pipette deposition. Arrayed nanodots allows multiplexing of assays, wherein multiple capture SOMAers with different

specificities can be exposed to individual samples. A diagram displaying the layout and a series of interactions in a Biodot LFT format, is shown in **Figure 5.15**.

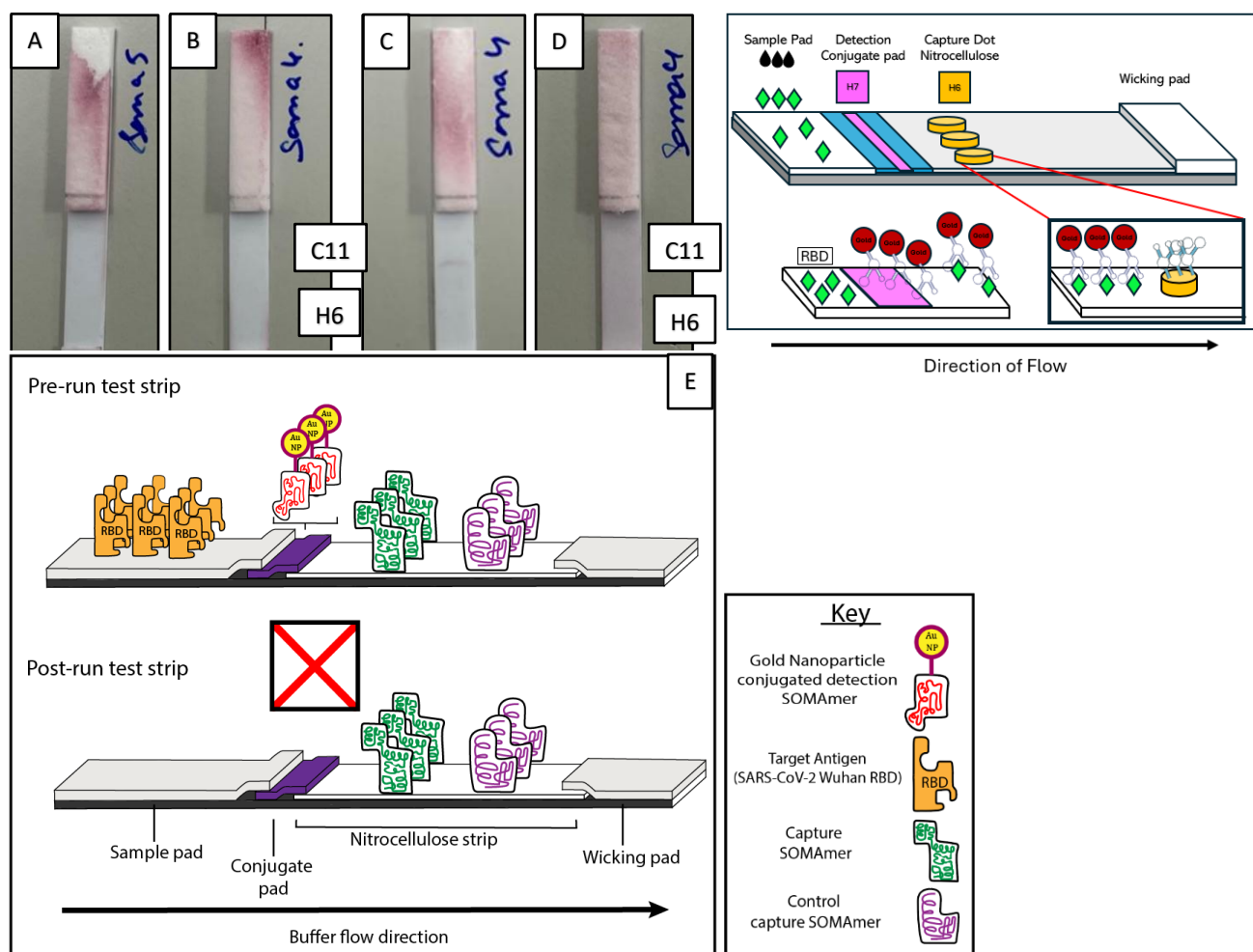


Figure 5.16: Binding of target antigen to dried detection SOMAmers and dried capture SOMAmers.

Two replicates of 20 ng, and three replicates of 40 ng of capture SOMAmers (H6 or C11) in 6 nL/replicate were applied with the Biodot dispenser to nitrocellulose strips and allowed to dry.

A. Desiccated Au-NP detection SOMAmer H7, B. Desiccated Au-NP detection SOMAmer H8

Conjugate pads were immersed in Au-NP detection SOMAmers at a final OD of 2 in dH₂O and subsequently allowed to dry before placing onto the nitrocellulose strip. 1.5 of SARS-CoV-2 Wuhan-Hu-1 RBD in 150 μ L of running buffer (100 μ M Z-Block, 4.5% BSA, 1 mM MgCl₂ in PBS) was applied to the sample pad and allowed to migrate by capillary action across the nitrocellulose strip to the wicking pad. Strips were read after 10 minutes. C – Pre-combined Au-NP detection SOMAmer H7 and SARS-CoV-2 Wuhan-Hu-1 RBD

D – Pre-combined Au-NP detection SOMAmer H8 and SARS-CoV-2 Wuhan-Hu-1 RBD

Au-NP detection SOMAmers to a final OD of 2 were pre-combined with 1.5 of SARS-CoV-2 Wuhan-Hu-1 RBD in 150 μ L of running buffer (100 μ M Z-Block, 4.5% BSA, 1 mM MgCl₂ in PBS). Pre-combined complexes were added to the sample pad and allowed to migrate by capillary action across the nitrocellulose strip to the wicking pad. Strips were read after 10 minutes. E – Illustration of pre- and post-run test components and binding structures, relevant to A and B. Evaluations performed on single replicate.

5.2.4.5 - Binding of target antigen to both dried detection SOMAmers and dried capture SOMAmers.

The next stage of development needed to move closer to a prototype LFT was to use a Biodot machine to apply capture and control SOMAmers H6 and C11, respectively to the nitrocellulose surface before drying. As described above, the use of Biodot deposited SOMAmers has the benefit of enabling future multiplexing of LFT assays but also reduces the quantity of capture SOMAmer by a factor of 2.5 and 5 compared with previous successful experiments (**Figure 5.14**). A further optimisation step investigated in this study, was that Au-NP conjugated detection SOMAmers H7 and H8 were applied separately to the conjugate pad and allowed to dry. This represented the use of the conjugate pad as a source of desiccated detection SOMAmer. The RBD, as the sample target antigen, was then diluted in running buffer and applied to the proximal end of the test strip. The buffer front migrated across the sample pad and over the immobilised capture SOMAmers towards the wicking pad by capillary action. Unfortunately, there were no visible dots indicating the expected tripartite interaction between capture SOMAmer (H6), RBD and pre-desiccated Au-NP conjugated detection SOMAmer (H7 or H8) (**Figure 5.16A and B**), as had been seen previously. This indicated that one of the interactions did not proceed as required. As an additional control experiment, pre-mixed RBD and Au-NP-conjugated detection SOMAmers (H7 and H8 separately) were applied in liquid form to the sample pad and migrated over the immobilised capture SOMAmers by capillary action (**Figure 5.16 C and D**). The purpose of this secondary experiment (which mimics that shown above) was to investigate the effect of reducing the quantity of capture SOMAmer, allowing a direct comparison between pipette deposition (750 μ L resulting in 100ng, as used in **Figure 5.14**) and Biodot deposition (6 μ L, resulting in 20 nG and 40 nG deposited herein).

Again, unfortunately there were no visible dots, indicating that the reduced quantity of capture SOMAmer potentially resulted in insufficient tripartite capture SOMAmer : RBD : Au-NP detection SOMAmer complexes to be visible.

5.2.4.6 - Titrating target antigen and increasing the quantity of detection SOMAmers.

Following the failure of (a) a reduced quantity of capture SOMAmer (20 nG or 40 nG Biodot, compared to 100 nG of pipette deposited) and (b) desiccated Au-NP conjugated detection SOMAmer, to enable visible binding, a further experiment was undertaken wherein the quantity of detection SOMAmer was increased four-fold (to an OD of 8) and not desiccated. As part of this study, the target antigen RBD was also titrated. The aim of these changes was to generate a positive result using the Biodot format which would form the basis of subsequent refinement experiments.

As previously, Biodots of the capture SOMAmers H6 and control C11 were applied to nitrocellulose and allowed to dry. Subsequently, pre-combined target antigen RBD at concentrations ranging from 50 µg/mL (7.5 µg total) to 0.00316 µg/mL (0.47 ng total) together with Au-NP conjugated detection SOMAmers (H7 and H8) were applied directly to the nitrocellulose to migrate by capillary action across the capture SOMAmers.

As can be seen in **Figure 5.17**, dots were clearly visible (outlined in yellow box), representing a tripartite interaction between capture SOMAmer H6, RBD at 50 µg/mL (7.5 µg total) and Au-NP conjugated detection SOMAmer at an OD of 8. Dots were also visible at RBD concentrations of 10 µg/mL (1.5 µg total) and 15.6 µg/mL (2.3 total), but not at lower RBD concentrations. As the RBD level of 1.5 was used in the previous experiment (**Figure 5.16**), this suggested that the quantity of RBD was not the primary limiting factor in the previous failure to generate visible tripartite

interactions. Therefore, it is assumed that increasing the quantity of Au-NP conjugated detection SOMAmer (to OD8) was the predominant factor in achieving a visible signal, although higher quantities of RBD also enhanced the signal.

There were no dots visible where the control capture SOMAmer C11 was used, indicating no non-specific interactions were present, even at the higher levels of RBD and Au-NP conjugated detection SOMAmers, all within the 10 minute run time. As the tripartite interactions successfully occurred with a Biodot-deposited capture SOMAmer at the same volume and concentration as the experiment showed in **Figure 5.16**, it can be assumed that capture SOMAmer abundance was not the limiting factor. It is possible however that the absence of a sample pad in this study, compared to the previous experiment (**Figure 5.16**), may have enhanced the quantity of available Au-NP-conjugated detection SOMAmer: RBD complexes migrating across the capture SOMAmer, enabling a visible signal to be generated.

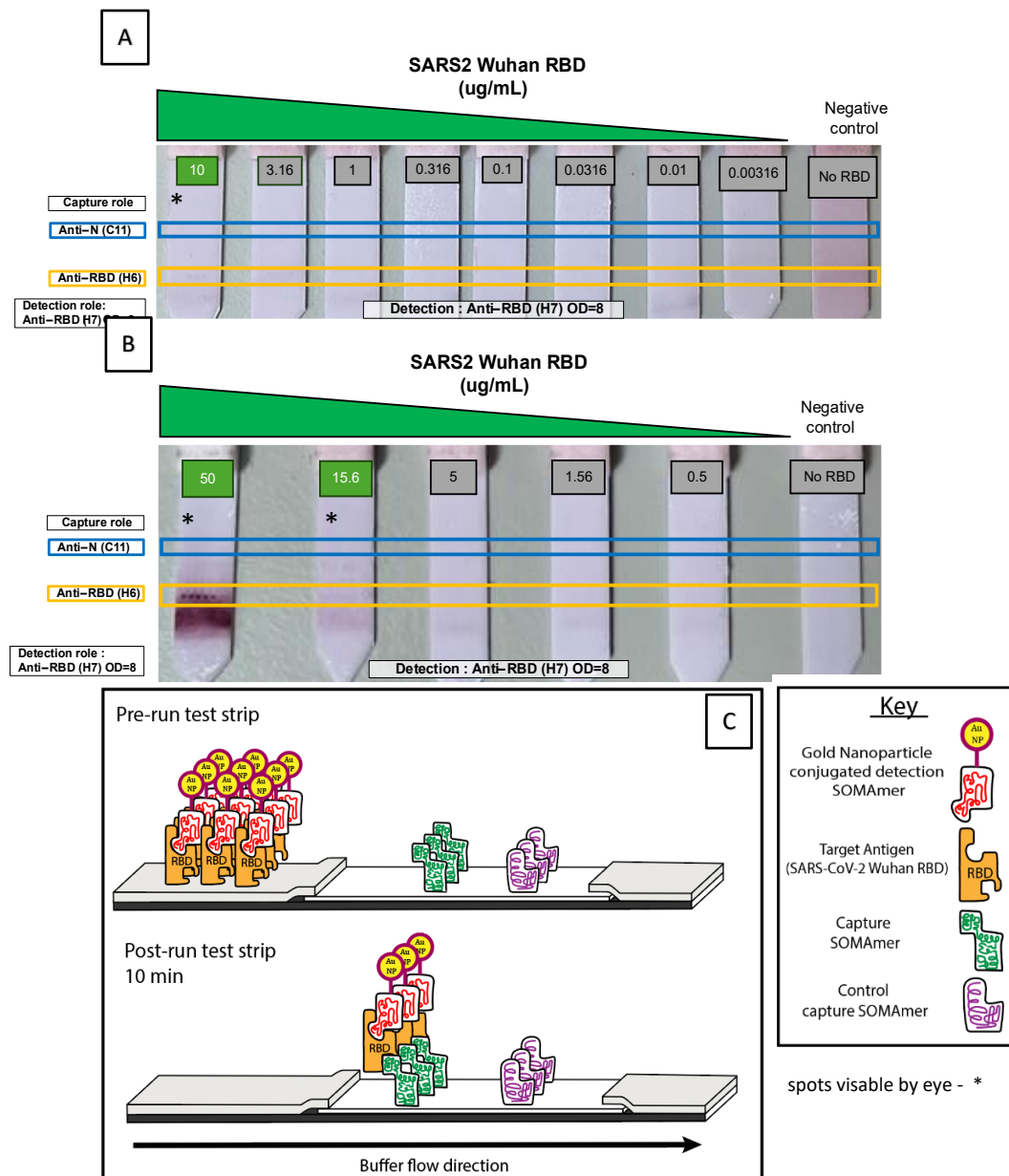


Figure 5.17: Titrating target antigen and using an increased quantity of detection SOMAmer.

The Biodot dispenser was used to spot two replicates of 20 ng, and three replicates of 40 ng of capture SOMAMers (H6 or C11) [6 nL/replicate] to the membrane before being allowed to dry.

The Au-NP detection SOMAmer (H7) at a final OD of 8, and pre-combined with a range of concentrations of SARS-CoV-2 Wuhan-Hu-1 RBD in 150 μ L of running buffer (100 μ M Z-Block, 4.5% BSA, 1 mM MgCl_2 in PBS) for 5 minutes, was subsequently added to a cuvette. RBD concentrations from 10 μ g/mL to 0.00316 μ g/mL (1.5 μ g to 0.47 ng) RBD concentrations from 50 μ g/mL to 0.5 μ g/mL (7.5 μ g to 0.75 μ g).

Nitrocellulose strips were placed into the cuvettes and buffer allowed to migrate up by capillary action, with strips read after 10 minutes.

Evaluations performed on single replicate.

C. Illustration of the expected pre- and post-run test components and binding structures, as described in A and B.

Faint positive test lines marked with an asterisk *.

5.2.4.7 - Non-specific binding interactions and the role of Z-block as a blocking agent

In our previous experiments, test strips were read 10 minutes after application of the sample, with dots clearly visible (**Figure 5.17** above, outlined in yellow box) representing a tripartite interaction between capture SOMAmer H6, an RBD and the Au-NP conjugated detection SOMAmer H7. However, it was observed that after a slightly longer period of 15 minutes incubation, dots were also observed with the nucleocapsid – specific control capture SOMAmer C11, indicating potential interactions with higher concentrations of RBD (50 μ g/mL to 10 μ g/mL) (an example is shown in **Figure 5.18A**).

Previous experiments had also demonstrated that it was necessary to increase the quantities of RBD antigen and Au-NP detection SOMAmer to generate a positive signal when using the Biodot deposited capture SOMAmer. Therefore, at least during this optimisation phase, reducing the quantities of these reagents to address the unwanted non-specific binding was not an option.

As an alternative, altering the concentration of Z-block was investigated. Theoretically, since there was likely to be a trade-off between increasing the quantity of Z-block and achieving the desired tripartite interactions, the concentration of RBD antigen was raised to 50 μ g/mL (7.5 μ g) from 10 μ g/mL (1.5 μ g). There was a trade-off between levels of Z-block and tripartite formation with incubation durations of 15 minutes. At the highest concentration of Z-block (500 μ M) there were no visible dots with either capture SOMAmer (H6 or C11) (**Figure 5.18B**). This suggests that non-specific tripartite reactions were prevented, but at the cost of desired specific tripartite reactions between H6:H7 and RBD. As the Z-block concentration was reduced from 500 μ M to 100 μ M, visible binding to capture SOMAmer H6 was restored. Also, non-

specific binding to capture SOMAmer C11 was absent. However, at the lower concentration of 25 μ M Z-block, both specific binding to capture SOMAmer H6 and non-specific binding (just visible) to capture SOMAmer C11 was apparent, indicating insufficient blocking. These results indicate the interrelatedness of interactions between components of the prototype LFT, and the requirement for an extensive iterative process to optimise performance as described in 5.1.4 above.

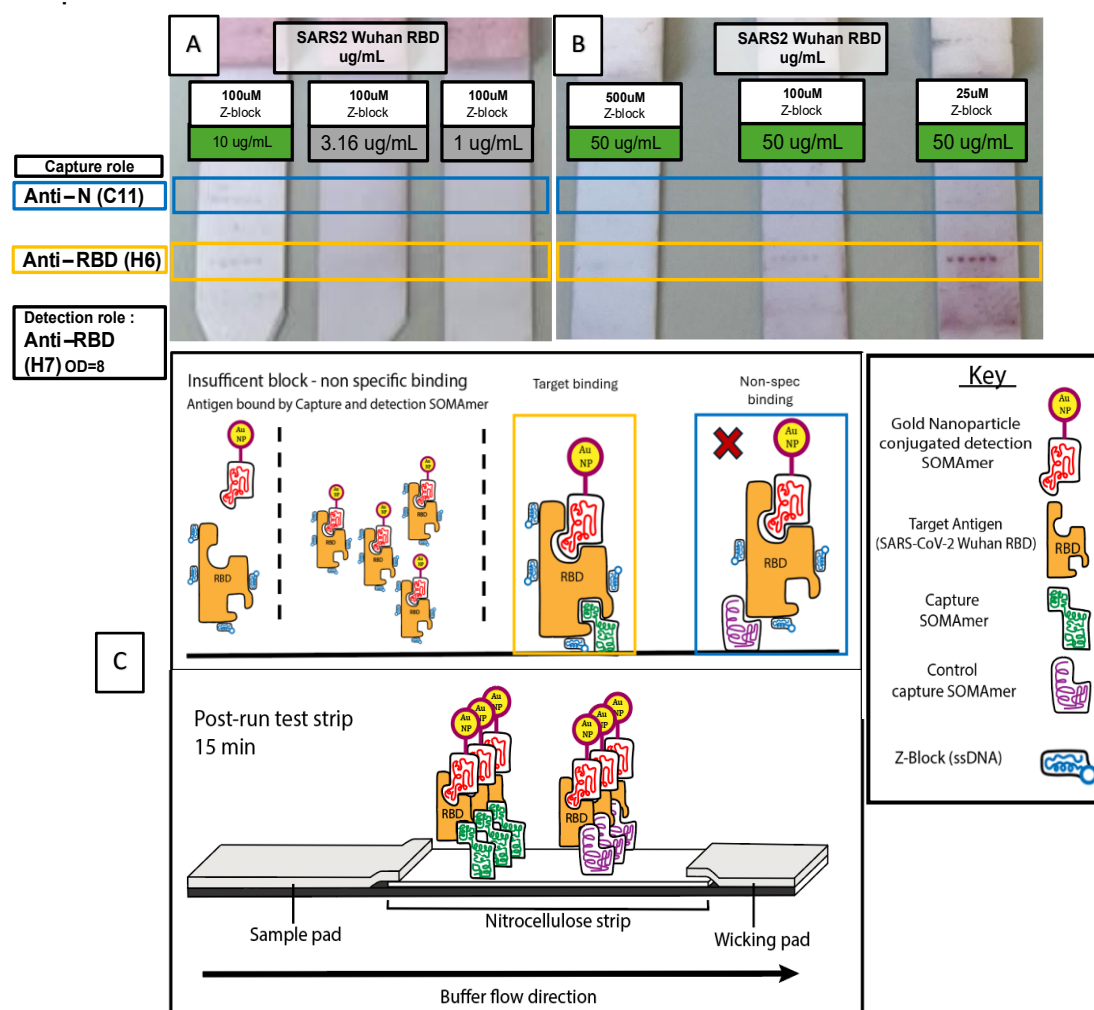


Figure 5.18: Non-specific binding at extended read times and an investigation of increasing Z-block concentration on non-specific binding.

A biodot dispenser was used to spot two replicates of 20 ng, and three replicates of 40 ng of each of the capture SOMAmers (H6 or C11) [6 nL/replicate] onto the membrane before being allowed to dry.

A. RBD concentrations from 10 $\mu\text{g/mL}$ to 1 $\mu\text{g/mL}$

Au-NP detection SOMAmer (H7) to a final OD of 8 was pre-combined with a range of concentrations of SARS-CoV-2 Wuhan-Hu-1 RBD in 150 μL of running buffer (100 μM Z-Block, 4.5% BSA, 1 mM MgCl_2 in PBS) for 5 minutes and subsequently added to a cuvette. Nitrocellulose strips were placed into cuvettes and buffer allowed to migrate up by capillary action and read after 15 minutes.

B. Z-block concentrations from 500 μM to 25 μM

Au-NP detection SOMAmer (H7) to a final OD of 8 was pre-combined with 50 $\mu\text{g/mL}$ of SARS-CoV-2 Wuhan-Hu-1 RBD (total 7.5 μg) in 150 μL of running buffer (4.5% BSA, 1 mM MgCl_2 in PBS) containing varying concentrations of Z-block for 5 minutes and subsequently added to a cuvette. Nitrocellulose strips were placed into cuvettes and buffer allowed to migrate up by capillary action and read after 15 minutes.

Evaluations performed on single replicate.

C. Illustration of post-run test components and binding structures after 15 minutes, relevant to A. Non-specific binding of Au-NP detection SOMAmer: RBD complexes to control capture SOMAmer C11 (binds SARS-CoV-2 Wuhan-Hu-1 nucleocapsid). Insufficient Z-block allows non-specific binding.

5.3 Discussion

The first priority in this chapter was to identify and select non-competing SOMAmer pairs to function as capture and detection molecules for a prototype LFT.

In the previous chapter, the SOMAmer library was sorted by ELONA into three groups, representing molecules with differing binding patterns to SARS-CoV-2 variant RBDs (subset shown in **Figure 5.1**). It was proposed that due to these separate ELONA binding patterns the SOMAmer epitopes were likely to be distinct to each of the three groups.

The distinct technical approach of applying the Carterra LSA SPR to assess which SOMAmers competed to bind epitopes within the RBDs of SARS-CoV-2 variants and wider sarbecoviruses was then used. Again, this analysis sorted the SOMAmers into groups with common RBD epitope binding sites (**Figure 5.7**). The groups were (a) H5:H6:H8:H9:H10, (b) H7:E7:E8 and (c) D4:E11. Non-competing pairs were chosen by taking a SOMAmer from different epitope groups.

Comparison of these two approaches in sorting SOMAmers into binding groups resulted in a strong degree of alignment, suggesting that the determination of SOMAmer binding sites was robust. Carterra LSA SPR determined epitope groups plotted onto **Chapter 4** ELONA data demonstrates the overlap in SOMAmer groupings (**Figure 5.19 and Table 5.2**). All SOMAmer binding determined by ELONA (green boxes) was confirmed by the presence of an epitope revealed by SPR analysis (shown as coloured dots). However, additional binding events were detected by SPR (coloured dots) that were not observed in ELONA (white boxes). This may be due to

the assay's greater analytical sensitivity, which allows lower levels of SOMAmer binding to be detected.

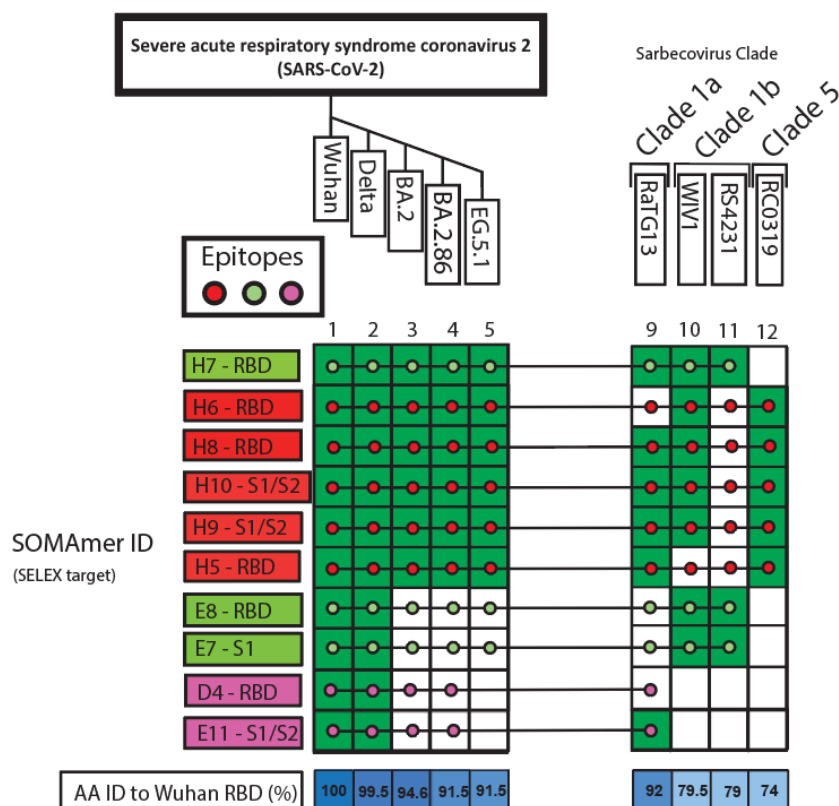


Figure 5.19: SPR determined epitope groups mapped to each SOMAmer.

SPR epitope analysis results (dots) displayed superimposed onto ELONA SOMAmer binding data (green squares for binding, white squares for no binding) exposed binding interactions between SOMAmer and RBD below the sensitivity of developed assay. Epitopes appear in RBDs not identified as ELONA binders.

	Pan variant binding (Subgroup 1)	SELEX target	Mutation limited binders (Subgroup 2)	SELEX target	Intermittent binders (Subgroup 3)	SELEX target
1	H7	RBD	E8	S1/S2	E9	RBD
2	H6	RBD	E7	S1	D5	RBD
3	H8	RBD	D4	RBD	D6	RBD
4	H10	S1/S2	E11	S1/S2	D7	RBD
5	H9	RBD	D3	S1	D2	S1
6	H5	RBD				

Table 5.2: RBD SOMAmer binding subgroups by ELONA and SPR-identified epitope groups.

SOMAmers were classified into subgroups 1, 2 and 3 according to their binding against the SARS-CoV-2 viral lineage, as shown in Chapter 4. Superimposed onto this classification are the epitope binding groups identified by SPR competition (SOMAmer labels highlighted in green, red and pink cells).

As part of the selection of SOMAmer pairs for taking forward into the prototype LFT, the molecular kinetics of association and dissociation of SOMAmers with target RBD antigen were investigated. The speed at which SOMAmer:RBD complexes form and break apart is important in the non-equilibrium system of an LFT format. Continued binding of complexes is required to enable a visible signal to be generated.

The kinetic performance of SOMAmers against a range of coronavirus RBDs was investigated by Carterra LSA SPR, and in many cases found to compare favourably with the kinetics of antibody and aptamer binding suitable for assay development (**Figure 5.11**). This gave confidence that chosen SOMAmer pairs for further development in an LFT format would have a suitable kinetic performance [138] . As discussed in the introduction section 5.1.4 above, multiple iterative steps are undertaken to optimise the multitude of interactions required to produce an effective LFT. Such steps may not be linear and the steps accomplished are shown in **Figure 5.21** below.

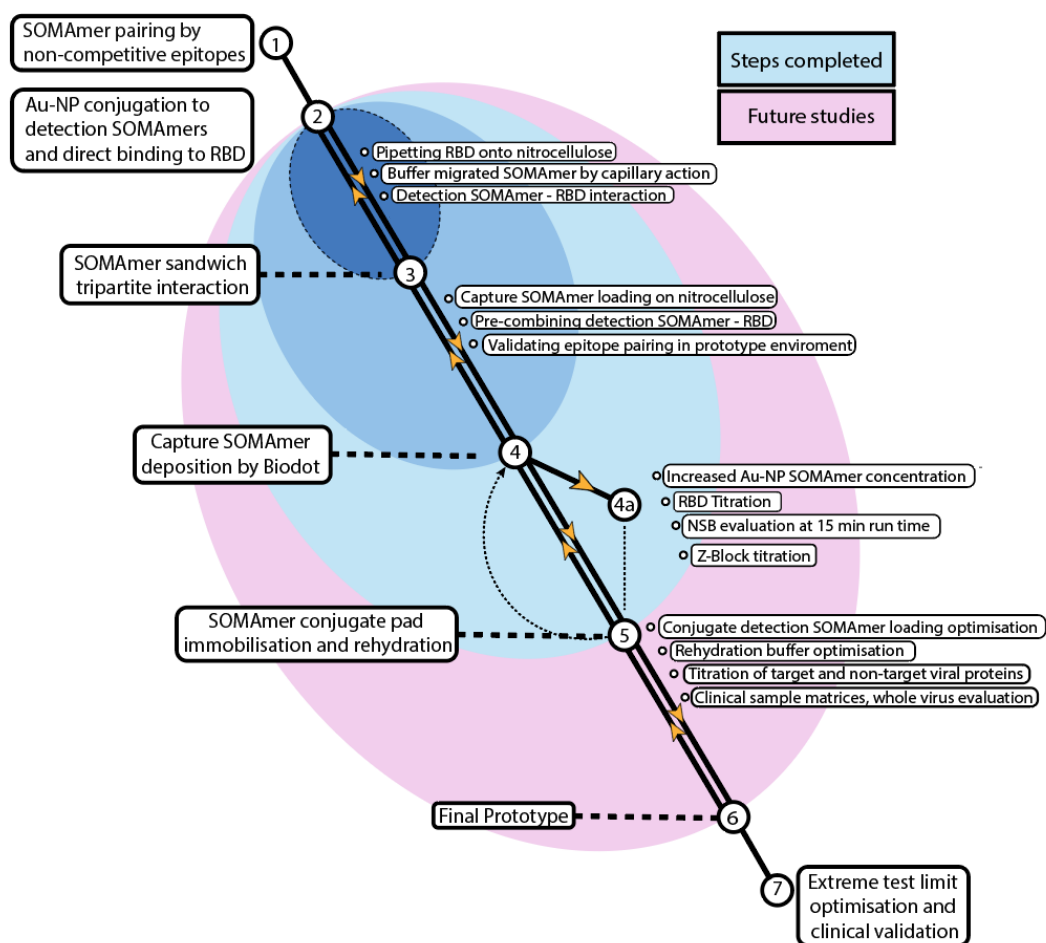


Figure 5.21: Iterative steps performed in LFD development towards creating a final prototype.

Stage of development are referenced in the text.

Blue highlighted ovals include steps completed as part of this study, which build on each other having established non-competing SOMAmer pairs (1). Au-NP detection SOMAmers functionality was established in (2), followed by incorporation into a sandwich tripartite format with capture SOMAmers (3). Capture elements were further refined in line with future multiplexing potential by micro deposition using Biodot machine (4) and conjugate pad immobilised detection SOMAmers. Non-specific binding (NSB) seen at extended run times was addressed (4a) with Z-block titration.

Studies carried out here have addressed some of these steps and have resulted in a combination of parameters resulting in successful specific tripartite interactions between SARS-CoV-2 Wuhan-Hu-1 RBD: detection SOMAmer: capture SOMAmer in a format where reagents are migrating across a nitrocellulose membrane.

Initial experiments showed that Au-NP-conjugated RBD detection SOMAmers could bind specifically to RBD immobilised onto a nitrocellulose membrane (**Figures 5.13 and 5.21**, step 2). This is not a situation that would occur in an LFT format, but was an important step in demonstrating binding following migration of detection SOMAmer across a nitrocellulose membrane.

The second step was closer to an LFT format, where capture SOMAmer was immobilised onto the nitrocellulose membrane, and pre-combined RBD: Au-NP-conjugated detection SOMAmer shown to successfully form tripartite complexes following migration in buffer (**Figures 5.14 and 5.21**, step 3). This validated the choice of non-competing SOMAmers following epitope analysis.

The next set of experiments proved too much of a challenge for the early prototype and required a step back. The reduction in bound capture SOMAmer following Biodot deposition, rather than pipette deposition and desiccation of Au-NP-conjugated detection SOMAmer onto the conjugate pad led to no visible tripartite interactions (**Figures 5.16 and 5.21**, step 4).

Taking a step back (**Figures 5.17 and 5.21**, step 4a), the next set of experiments reverted to pre-combined RBD: Au-NP detection SOMAmers to circumvent any technical barriers with detection SOMAmer rehydration, which had not been fully investigated and optimised. A range of concentrations of RBD antigen and increased quantity of Au-NP-conjugated detection SOMAmer were pre-combined and run across Biodot deposited capture SOMAmer, which resulted successfully in visible dots at

higher concentrations of RBD. This indicated that the reduced quantities of capture SOMAmer due to Biodot deposition were able to produce visible tripartite interactions in the presence of sufficiently high quantities of RBD: Au-NP detection SOMAmer complexes. In all experiments carried out to this point, there were no tripartite interactions formed with the control capture SOMAmer C11 which binds to nucleocapsid, indicating the absence of non-specific interactions when samples were run for 10 minutes. However, when reactions on nitrocellulose strips were allowed to continue for a slightly longer period, some non-specific binding to capture SOMAmer C11 was seen. This was addressed by increasing the quantity of blocking reagent, Z-block (**Figures 5.18 and 5.21**, step 4a). However there was a trade off where excessive concentrations of Z-block reduced successful formation of tripartite complexes. This early set of experiments demonstrated that specific tripartite complexes could be formed in a format where reagents are migrating across a nitrocellulose membrane to produce a visible signal. Future studies would be required to replicate these experiments extensively to ensure results are robust and reproducible. Limited reagent availability during prototype development reduced the number of replicate experiments that could be undertaken.

Further studies are also required to investigate and resolve the rehydration of detection SOMAmer from the conjugate pad to enable tripartite complexes to be formed entirely within the liquid migration phase.

High concentrations of RBD antigen were required to demonstrate visible tripartite complex formation in these early experiments. Again, further studies would be required to investigate optimisation of all components to improve antigen sensitivity, or enhanced reading of reactions invisible by eye. However the currently detectable antigen levels are outside the range that would be found in a real world sample.

There are a small number of publications describing the use of aptamers as binding molecules in lateral flow type devices for detection or diagnostic purposes. These are laboratory studies and there is limited evidence of progression to a commercial product. Some of the assay conditions and sensitivity data in these publications is shown below in **Table 5.3**. The studies used spike protein as the target antigen and assigned a limit of detection (LOD) according to the lowest detectable quantity of antigen, which ranged in these publications from 1.8 ng to 10 ng. Even though the spike protein is approximately ten times larger in mass than the RBD, this range of published LODs is approximately 1000 fold lower than was detected in this study (1.5 of RBD).

Interestingly, the best performing assay [138] required target antigen and detection aptamer to be pre-incubated together in liquid format before application to the lateral flow device. This does not replicate the ideal LFT format where detection molecules are desiccated in the conjugate pad. However, this aptamer sandwich lateral flow assay (Aptaflow) was able to detect UV-inactivated virus of 10^6 copies/mL, which is the minimal LOD set by the WHO [139]. The sensitivity of the Aptaflow format was tested with UV-inactivated virus and recombinant full length spike protein. The LOD of recombinant spike protein was shown to be considerably higher ($\sim 6 \times 10^9$ copies of spike protein) than intact virions ($\sim 2 \times 10^6$ copies of spike protein) [140].

This higher sensitivity may be due to the higher detection aptamer binding capacity of virus particles, which contain multiple copies of the relevant epitope, therefore binding more detection aptamers leading to a higher visible signal. This compares with detection aptamer interaction with target antigen containing potentially three epitopes for a spike trimer. Therefore, the Yang (2022) study highlights the importance of assessing LOD performance with whole virus (and potentially virus associated

proteins) as would be found in a diagnostic sample, rather than just recombinant protein alone. Target LODs based exclusively on calculations of recombinant protein mass, replicates of molecules per virion and sample virus load may be too stringent in LFT evaluations. The published studies were carried out with a range of different conditions in terms of buffers, incubation times, reagent quantities, etc so comparisons with the current study are difficult to draw. However, the difference in sensitivity may reflect variable aptamer/SOMAmer performance with target antigen, as well as potentially sub-optimal prototype conditions. However, aptamers/SOMAmers compare poorly with LFTs based on Mabs as capture and detection MREs in terms of lower limits of detection. There are very few commercial Mab-based LFTs detecting spike protein, with the predominant tests being based on SARS-CoV-2 nucleoprotein detection. As an example, a nucleoprotein -based LFT showed an LOD of 8 pg, which is approximately 1000 times lower than aptamer-based tests as shown in **Table 5.3**.

[141]

Year published	Aptamer designation	Running/Binding buffer	Assay analyte	LOD as published	LOD (ng)	Ref
2023s	SNAP1- gold NP and SNAP4.74- biotinylated aptamer (LFD) 25 nM biotinylated aptamer, and 50 µL AU-NP aptamer	1% BSA, 0.1 mg/mL tRNA, 0.1 mg/mL SS DNA, 0.01% Tween-20) 100 µL total volume	Spike S1	100pM Spike (approx. 6×10^9 Spikes) 10^6 viral copies/mL (approx. to 2×10^6 Spikes)	(100 pM) 1.8 ng	[138]
2025	Aptamer (LFD)	PBS 10 mM pH 7.4) 2 % (w/v) sucrose, 0.5 % (v/v) Tween-20 1 % (w/v) BSA dried before use	Spike	0.1–1 µg/mL with an LOD of 51.81 ng/mL	5.2 ng	[142]
2022	Aptamer (LFD)	0.1 M PBS, 1 % BSA, 0.1 % Triton ×100 100 µL total volume	Spike	5 µL of 2.0 ng/µL	10 ng	[143]
2021	SNAP1.5	-	Spike	250 pM 2.5×10^7 copies/mL	4.5 ng	[144]

Table 5.3: Published studies describing assessment of aptamers in lateral flow formats with visible endpoints.

Running buffer conditions, target antigen and limit of detection (LOD) are shown, with LODs expressed in ng for comparison.

Chapter 6: General discussion and future directions

The detection and diagnosis of infectious disease is essential in its control at both the population level and for an affected individual. Understanding trends and outbreaks of infectious disease allows governments and animal and health structures to prepare and respond to mitigate public health, economic and social impacts. Accurate and timely infectious disease diagnosis for the patient or affected animal enables optimal treatment to restore health and actions to reduce the risk of onward transmission. In animal health, diagnostics are key to enabling demonstration of disease freedom in populations allowing freedom of movement, success of disease eradication programmes as well as interventions [145].

Understanding of the critical role of diagnostics was heightened by the recent COVID-19 pandemic where self-administered, easy to use diagnostics became part of the public's daily lives [72, 83]. At the individual's level, a positive diagnosis of COVID-19 allowed decisions to be made about self-isolation which had the effect of interrupting transmission. At the population level, diagnostic data informed case numbers, circulating viral strains and the effects of social interventions and vaccination programmes.

In animal health, the UK notifiable infection, Foot and Mouth Disease (FMD) exemplifies how diagnostic confirmation, rather than reliance on visible disease transmission within a herd, is central to informing timely control measures. Control of FMD uses molecular and non-molecular diagnostic techniques in line with DEFRA and WOAH guidance [145]. Interventions include culling of infected animals and restriction of animal movement between sites, until freedom from disease is demonstrated.

Following the severe impacts of the COVID-19 pandemic, the development of the 100 Days Mission [6] identified the ambitious target of ensuring supply of diagnostics, vaccines and therapeutics within 100 days of the identification of a pandemic threat. Numerous challenges have been identified in the goal of rapidly producing diagnostics at scale [7], some of which are technical, such as time taken to generate reagents, validate tests and scale up production. Most rapid antigen detection assays depend on monoclonal antibodies as specific recognition elements, but these reagents take many weeks to months to develop.

The WHO established desirable criteria for diagnostics with the acronym REASSURED [9]: real-time connectivity, affordable, sensitive, specific, user-friendly, rapid and robust. A trade-off exists between the accuracy, accessibility and affordability of diagnostic tests, leading to rapid diagnostic tests being most commonly used by primary healthcare, community health workers, and the wider population. An illustration of diagnostic test compared with sensitivity is shown in **Figure 6.1**, where data was derived from policy and guidance documents. The rapid antigen test (LFT), although less sensitive, is the most affordable.

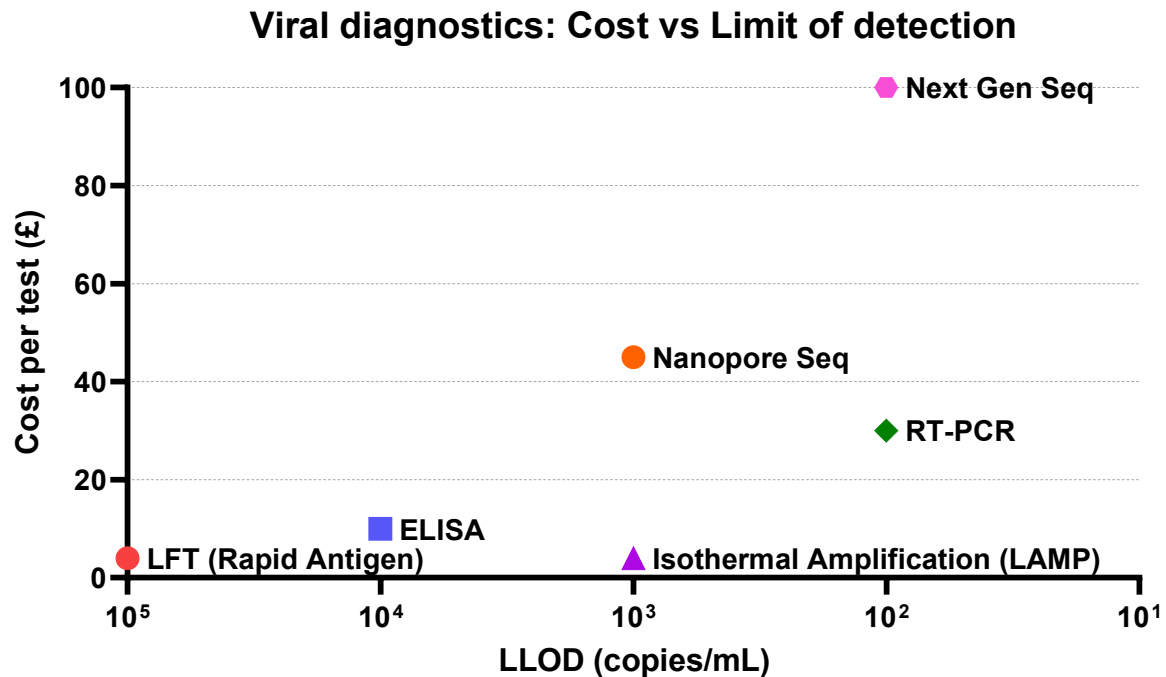


Figure 6.1: Trade-off of diagnostic test sensitivity versus cost.
 Illustration based on data derived from policy and guidance documents.
 [146, 147]

The choice of diagnostic technology depends on its purpose, for example high specificity techniques such as RT-PCR or sequence analysis are required for confirmatory diagnostics and reference evaluation. In contrast, screening assays are used for surveillance analysis to investigate populations of apparently healthy individuals to identify if any individuals are infected. These assays are characterised by speed, affordability and sensitivity, and include serology tests, RT-PCR, and rapid antigen-based tests.

Screening of host populations to detect and monitor circulating viruses is enabled by diagnostic methods that can detect a viral family-wide range. An example of this approach is a pan-coronavirus PCR assay capable of detecting all genera of CoVs, which can be used to screen wildlife reservoirs for viral prevalence and inform spillover risk [75]. Conserved sequences and resulting epitopes across viral families enable

broad detection approaches useful for surveillance and epidemiology. In addition, conserved sequences enhance resistance to mutation rendering diagnostic tests ineffective. Alternatively, a sequencing-based approach to surveillance has the advantages of being unbiased in identifying novel pathogens and provides more detailed information than PCR.

A further example of the potential utility of a pan-virus family diagnostic was reported by Yang et al [148], where a universal capture ligand and mAb-based assay able to detect all seven serotypes of FMDV was proposed as useful in the field as a rapid diagnostic. Such an assay may be useful for on-site detection of disease whilst laboratory-based confirmatory diagnostic assays are underway.

In contrast, specific diagnostic tests determine the presence of individual viral species which may inform patient management and wider public or veterinary health interventions. In order to achieve test specificity, diagnostic assays such as PCR are based on viral regions unique to the target, but continued vigilance is required to ensure viral mutation does not render the recognition elements redundant.

The research objectives for these studies were set out in **Chapter 1**, and all have been addressed. In the first objective, one of the aims of this study was to investigate the range of coronaviruses that SOMAmers could detect as a basis for a potential screening assay or a variant specific diagnostic. The data presented in this study investigated SOMAmer binding ability by ELONA (as developed in **Chapter 3**) to twenty-seven recombinantly expressed RBD or full-length spike proteins from three genera of the *Coronaviridae*, starting with SARS-CoV-2 variants and expanding out to wider sarbecoviruses and other alpha-, beta- and gammacoronaviruses.

The library of 45 SOMAmers was based on a SELEX process using SARS-CoV-2 Wuhan-Hu-1 RBD, S1 and S1/2, and 16 of these SOMAmers bound by ELONA to a

varying range of SARS-CoV-2 variant RBDs, diminishing according to phylogenetic evolution and reducing RBD amino acid homology to Wuhan-Hu-1 (**Chapter 4: Figures 4.2, 4.4 and 4.11**). A sub-group of SOMAmers (pan-variant binders) bound to all the SARS-CoV-2 variants tested, suggesting more conserved epitopes were recognised. Interestingly, another sub-group of SOMAmers (mutation-limited binders) recognised SARS-CoV-2 variant RBDs up to a stage in phylogenetic evolution when Omicron emerged, suggesting loss of epitopes (**Chapter 4, Figure 4.13**). Emergence of the Omicron variants showed a marked evolution from ancestral strains, enhancing immune evasion and tropism for nasal epithelium which depends on the spike protein [149]. Amino acid alignment of RBD sequences highlighting key changes between SARS-CoV-2 Alpha and the Omicron variants BA.1 and BA.2 may indicate areas associated with changes in RBD structure leading to loss of epitopes for these SOMAmers (**Chapter 4, Figure 4.15**).

Investigation of the range of binding of the SARS-CoV-2 RBD SOMAmers, including those from the SOMAmer second library (A1, A2, A3), to a wider set of virus RBDs from across the sarbecovirus clades showed a varied pattern of binding (**Chapter 4, Figures 4.6, 4.7 and 4.11**). The most widely recognised sarbecovirus by these SOMAmers was Clade 1b RaTG13, which shares 92% amino acid homology with SARS-CoV-2 Wuhan-Hu-1 RBD, and both viruses use the human ACE2 receptor. All sarbecovirus clades tested were recognised by one or more SOMAmers, with clade 5 Rc-o319 and clade 1a WIV-1 RBDs bound by higher numbers of SOMAmers. There did not appear to be strong alignment of SOMAmer binding with sarbecoviruses that use human ACE2 as a receptor. Examination of binding patterns of SOMAmers to wider alpha-, beta- and gammacoronaviruses full length spike proteins showed limited

recognition by a small number of SOMAmers with the betacoronavirus HKU1 and the alphacoronavirus 229E.

One of the aims of these studies was to determine if SOMAmers were capable of recognising individual species or a broad range of coronaviruses. A few SOMAmers (D5, D7, D4 and D3; **Chapter 4: Figures 4.2, 4.4 and 4.11**), recognised only SARS-CoV-2 variants (but not all variants tested) illustrating binding to species-specific epitopes. There was a progressive loss of binding across 27 viruses with increasing phylogenetic distance from SARS-CoV-2 moving across variants, then wider sarbecoviruses and wider viral genera of alpha-, beta- and gammacoronaviruses. This underscores the challenge of developing broadly cross-reactive affinity reagents and highlights the structural limits of SARS-CoV-2 Wuhan-Hu-1-centred SELEX selection strategies.

A requirement for development of an LFT prototype was the identification of pairs of SOMAmers with non-competing epitopes to fulfil the roles of capture and detection molecules for the antigen of interest. The categorisation of binding to SARS-CoV-2 variants RBDs by ELONA inferred separate groupings of SOMAmers with common binding ranges. Epitope binning by Catterra LSA SPR indicated groups of SOMAmers with common epitope sites on SARS-CoV-2 RBD, which aligned with the ELONA data. This allowed SOMAmer pairs to be chosen from separate epitope groups that did not compete, to be taken forward for LFT prototype development.

Initial LFT prototype development experiments demonstrated that Au-NP-conjugated RBD detection SOMAmers could specifically bind to RBD immobilized on a nitrocellulose membrane, validating the detection method (**Chapter 5, Figures 5.13 and 5.21**, step 2). The next phase involved immobilizing capture SOMAmer and allowing binding with pre-combined RBD: Au-NP-conjugated detection SOMAmer,

successfully forming tripartite complexes in a format resembling LFT (**Chapter 5, Figures 5.14 and 5.21**, step 3).

Challenges arose with the prototype due to decreased quantities of desiccated capture SOMAmer using Biodot deposition, which hindered visible tripartite interactions (**Chapter 5, Figures 5.16 and 5.21**, step 4). To address this, experiments reverted to pre-combined RBD: Au-NP detection SOMAmers, resulting in visible dots at higher RBD concentrations and showing that lower capture SOMAmer quantities could still produce visible interactions when sufficient RBD: Au-NP complexes were present.

No tripartite interactions were observed with control capture SOMAmer C11, indicating minimal non-specific interactions, although some were detected with extended reaction times. Increasing the blocking reagent, Z-block, helped reduce non-specific binding but at the cost of tripartite complex formation (**Chapter 5, Figures 5.18 and 5.21**, step 4a). Overall, these experiments showed that specific tripartite complexes could form on a nitrocellulose membrane, but more studies are needed for robustness and reproducibility.

There are few publications detailing aptamers as binding molecules in lateral flow devices for detection or diagnostics. Most studies are laboratory-based with limited progression to commercial products, although the literature does not record reasons for not progressing. The literature describes options for alternative reporting label technologies to AuNP, such as silica nanoparticles, magnetic separation and enzyme amplification. This may suggest that commercial development is constrained due to inadequate sensitivity. [108]

The aptamer-based LFT assays described in **Chapter 5, Table 5.3** primarily targeted spike protein, reporting limits of detection (LOD) ranging from 1.8 ng to 10 ng, significantly lower than the 1.5 µg of receptor-binding domain (RBD) detected in this study.

Notably, one assay required pre-incubation of the antigen with the detection aptamer before application, deviating from the ideal lateral flow test (LFT) format. This aptamer sandwich lateral flow assay (Aptaflow) detected UV-inactivated virus at 10^6 copies/mL, meeting WHO's minimum LOD. However, the LOD for recombinant spike protein was considerably higher ($\sim 6 \times 10^9$ copies) compared to intact virions ($\sim 2 \times 10^6$ copies). This discrepancy may arise from the virus's multiple epitopes enhancing the binding of detection aptamers, unlike recombinant proteins with fewer epitopes. The variances in sensitivity across studies may reflect differences in aptamer performance, target antigens, and experimental conditions, complicating direct comparisons.

Future studies to develop the LFT prototype based on our SOMAmer library would need to increase the test sensitivity in the first instance, which would require an iterative optimisation of all interactions and components as outlined in **Chapter 5, Figures 5.3 and 5.21**. If this could be achieved, the next step would be to establish desiccation and rehydration of capture SOMAmer in the LFT format to simplify use. Following successful establishment of an LFT prototype, evaluation with a range of sample types, validation of specificity/ sensitivity and real-world trials would be required according to WHO TPP guidelines.

This study investigated the potential for molecular recognition elements, SOMAmers, to substitute for monoclonal antibodies in a rapid diagnostic test (LFT). There are many advantageous features of SOMAmers compared with mAbs, such as cost and speed of production, use of animal-free systems and thermal stability. The SOMAmers

studied here showed a broad binding range against divergent coronaviruses compared the SELEX agent used in their generation, SARS-CoV-2 Wuhan-Hu-1. This broader binding range could be an advantage depending on the purpose of an assay, although mAbs may also have this characteristic.

These attributes are countered by some limitations of SOMAmers. A high degree of non-specific binding in ELONA was addressed with extensive blocking with a polyanionic competitor, Z-block, and washing to ensure off-target binding was diminished. SOMAmer non-specific binding was also problematic in LFT prototype development, with a trade-off between blocking to achieve specificity and test sensitivity. These constraints in sensitivity and specificity may prove to be barriers to the commercial development of SOMAmers in LFDs.

The potential for enhancing SOMAmer performance in the LFTs includes a SELEX process against a more abundant viral target in the respiratory tract during infection, such as coronavirus nucleocapsid rather than spike protein, which may enhance test sensitivity. In addition, depending on the requirement for a pan-coronavirus or a species-specific assay, the SELEX target used for SOMAmer generation could be based on a more conserved, or more diverse protein respectively. To achieve a SOMAmer library with more specific binding properties, further rounds of counter-selection with closely related off-target proteins could be undertaken to remove non-specific binders. For future diagnostic MREs, SARS-CoV-2 nucleoprotein as a target would offer the benefits of abundance and a high level of conservation leading to increased sensitivity and longer term clinical application respectively.

In future, it may be possible to rationally design SOMAmers in silico with binding recognition specific to chosen epitopes using an AI and bioinformatics approach. Currently, methodologies based on predicting folded aptamer structures and their

binding interactions with other molecules are developing. For example, Ochoa and Milam [150] recently demonstrated the ability of AlphaFold3 to accurately predict aptamer structures and interactions with target protein which had been previously resolved experimentally.

However, there are currently limited SOMAmer-specific models to predict structure as there is a scarcity of data for experimentally solved structures [151]. Modifications to amino acid side chains present on SOMAmers, compared with the simpler nucleic acid-only structures of aptamers complicate interpretations and a defined pathway for modelling is yet to be determined. This situation may change as future versions of prediction software such as AlphaFold develop as an increasing number of experimentally solved SOMAmer structures are published compared to 200k+ protein structures used in development of the Alphafold system [152].

The future may hold technical approaches that enable rapid rational design and manufacture of molecular recognition agents, such as SOMAmers, which could be used for therapeutic and diagnostic applications. This could play a significant role in pandemic preparedness and an urgent response to an outbreak or infectious disease emergency.

Appendices

Appendix 1: SOMAmer library with associated SELEX target protein and subdomain.

Well	SELEX Target	Tag	Well	SELEX Target	Tag
A4	Nucleoprotein_SARS	Amine Group	C7	Spike glycoprotein (S1 S2 ECD)	Amine Group
C11	Nucleoprotein_SARS	Biotinylated	F2	Spike glycoprotein (S1 S2 ECD)	Biotinylated
F11	Nucleoprotein_nCov-2	Amine Group	C6	Spike glycoprotein (S1 S2 ECD)	Amine Group
G8	Nucleoprotein_nCov-2	Biotinylated	F1	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A1	Nucleoprotein_SARS	Amine Group	C5	Spike glycoprotein (S1 S2 ECD)	Amine Group
C8	Nucleoprotein_SARS	Biotinylated	E12	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			C4	Spike glycoprotein (S1 S2 ECD)	Amine Group
C2	Spike glycoprotein (RBD)	Amine Group	E11	Spike glycoprotein (S1 S2 ECD)	Biotinylated
E9	Spike glycoprotein (RBD)	Biotinylated	C3	Spike glycoprotein (S1 S2 ECD)	Amine Group
C1	Spike glycoprotein (RBD)	Amine Group	E10	Spike glycoprotein (S1 S2 ECD)	Biotinylated
E8	Spike glycoprotein (RBD)	Biotinylated	B11	Spike glycoprotein (S1 S2 ECD)	Amine Group
A12	Spike glycoprotein (RBD)	Amine Group	E6	Spike glycoprotein (S1 S2 ECD)	Biotinylated
D7	Spike glycoprotein (RBD)	Biotinylated	E5	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A11	Spike glycoprotein (RBD)	Amine Group	B10	Spike glycoprotein (S1 S2 ECD)	Amine Group
D6	Spike glycoprotein (RBD)	Biotinylated	E4	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A10	Spike glycoprotein (RBD)	Amine Group	B9	Spike glycoprotein (S1 S2 ECD)	Amine Group
D5	Spike glycoprotein (RBD)	Biotinylated	B8	Spike glycoprotein (S1 S2 ECD)	Amine Group
A9	Spike glycoprotein (RBD)	Amine Group	E3	Spike glycoprotein (S1 S2 ECD)	Biotinylated
D4	Spike glycoprotein (RBD)	Biotinylated	B7	Spike glycoprotein (S1 S2 ECD)	Amine Group
F8	Spike glycoprotein (RBD)	Amine Group	E2	Spike glycoprotein (S1 S2 ECD)	Biotinylated
H7	Spike glycoprotein (RBD)	Biotinylated	E1	Spike glycoprotein (S1 S2 ECD)	Biotinylated
F9	Spike glycoprotein (RBD)	Amine Group	B6	Spike glycoprotein (S1 S2 ECD)	Amine Group
H6	Spike glycoprotein (RBD)	Biotinylated	B4	Spike glycoprotein (S1 S2 ECD)	Amine Group
F10	Spike glycoprotein (RBD)	Amine Group	D11	Spike glycoprotein (S1 S2 ECD)	Biotinylated
H5	Spike glycoprotein (RBD)	Biotinylated	B5	Spike glycoprotein (S1 S2 ECD)	Amine Group
			D12	Spike glycoprotein (S1 S2 ECD)	Biotinylated
B12	Spike glycoprotein (S1 subunit)	Amine Group	B3	Spike glycoprotein (S1 S2 ECD)	Amine Group
E7	Spike glycoprotein (S1 subunit)	Biotinylated	D10	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A8	Spike glycoprotein (S1 subunit)	Amine Group	B2	Spike glycoprotein (S1 S2 ECD)	Amine Group
D3	Spike glycoprotein (S1 subunit)	Biotinylated	D9	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A7	Spike glycoprotein (S1 subunit)	Amine Group	D8	Spike glycoprotein (S1 S2 ECD)	Biotinylated
D2	Spike glycoprotein (S1 subunit)	Biotinylated	B1	Spike glycoprotein (S1 S2 ECD)	Amine Group
A6	Spike glycoprotein (S1 subunit)	Amine Group	F3	Spike glycoprotein (S1 S2 ECD)	Amine Group
D1	Spike glycoprotein (S1 subunit)	Biotinylated	H12	Spike glycoprotein (S1 S2 ECD)	Biotinylated
A5	Spike glycoprotein (S1 subunit)	Amine Group	FA	Spike glycoprotein (S1 S2 ECD)	Amine Group
C12	Spike glycoprotein (S1 subunit)	Biotinylated	H11	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			F5	Spike glycoprotein (S1 S2 ECD)	Amine Group
			H10	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			F6	Spike glycoprotein (S1 S2 ECD)	Amine Group
			H9	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			F7	Spike glycoprotein (S1 S2 ECD)	Amine Group
			H8	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			H4	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G7	Spike glycoprotein (S1 S2 ECD)	Amine Group
			G6	Spike glycoprotein (S1 S2 ECD)	Amine Group
			H3	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G1	Spike glycoprotein (S1 S2 ECD)	Amine Group
			H2	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			H1	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G5	Spike glycoprotein (S1 S2 ECD)	Amine Group
			G4	Spike glycoprotein (S1 S2 ECD)	Amine Group
			G12	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G3	Spike glycoprotein (S1 S2 ECD)	Amine Group
			G11	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G10	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			G2	Spike glycoprotein (S1 S2 ECD)	Amine Group
			G9	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			F12	Spike glycoprotein (S1 S2 ECD)	Amine Group
			C10	Spike glycoprotein (S1 S2 ECD)	Biotinylated
			A3	Spike glycoprotein (S1 S2 ECD)	Amine Group
			A2	Spike glycoprotein (S1 S2 ECD)	Amine Group
			C9	Spike glycoprotein (S1 S2 ECD)	Biotinylated

Appendix 2: Virus accession number references

Sequences sourced from NCBI Genbank (<https://www.ncbi.nlm.nih.gov/genbank>) and NCBI RefSeq databases ([RefSeq: NCBI Reference Sequence Database](#)).

Virus species	Variant	Spike protein accession number
SARS-CoV-2	Wuhan	YP_009724390.1
SARS-CoV-2	Alpha	A0A7T8KZF1
SARS-CoV-2	Beta	QRN78347
SARS-CoV-2	Delta	QUD52764.1
SARS-CoV-2	Gamma	QRX39425.1
SARS-CoV-2	BA.1	UFO69279.1
SARS-CoV-2	BA.2	UJP23605.1
SARS-CoV-2	SARS2 S RBD (BA.4)	UPP14409.1
SARS-CoV-2	SARS2 S RBD (BA.5)	(UOZ45804.1)
SARS-CoV-2	XBB	YP_009724390.1
SARS-CoV-2	BA.2.86	YP_009724390.1
SARS-CoV-2	JN.1	YP_009724390.1
SARS-CoV-2	EG.5.1	YP_009724390.1
SARS1	-	YP_009825051.1
RP3	-	AAZ67052.1
HKU9	-	YP_001039971.1
HKU1	-	YP_173238.1
MHV	-	NP_045300.1
Longquan RI rat	-	AID16716.1
OC43	-	YP_009555241.1
BCoV	-	NP_150077.1
MERS	-	YP_009047204.1
PRCV	-	ABG89317
NL63	-	YP_003767.1
229E	-	YP_002308227.1
IBV	-	YP_009825051.1
RaTG13	-	QHR63300.2
RCo319	-	QHR63308.1
RsSHC014	-	AGZ48806.1
WIV1	-	AGZ48828.1
RS4231	-	AIA62300.1
BtKY72	-	YP_009824989.1
RhGB07	-	UFO69203.1
RacCS203	-	QOI60332.1
RF1	-	ABL12357.1
RS4081	-	AIA62294.1

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