# SARS-CoV-2 (2019-nCoV) vaccine

**Rapid Deployment Vaccine Collaborative (RaDVaC)**

**Preston Estep, Don Wang, Alex Hoekstra, Ranjan Ahuja, George Church, UV, SC, MR, TE, SE, RL, ML, RH, KL, LM, EB, HS, LB, GG, MG, FG, RG, MG et al.**

# Table of Contents

## TERMS OF USE/INFORMED CONSENT (last updated 8/16/20)

This document describes the rationale, design, formulation, and self-administration of a vaccine for SARS-CoV-2.  By using this information you agree to the following: 1) you are a consenting adult (in the USA, at least 18 years of age) and 2) to take full responsibility for your use of information, vaccine or materials, including redistribution, modification, vaccine formulation, production and administration.

The rapid vaccine development and deployment strategy of RaDVaC is founded upon the core principle of rapid updating of vaccine designs, based on newly published information from the forefront of biomedical research. This agile approach has the potential to produce better vaccines much more quickly than traditional approaches; but such rapid advances are incompatible with the usual clinical trial requirements of a fixed vaccine design throughout the lengthy sequence of trials, and Institutional Review Board (IRB) review and approval (due to shifting vaccine design and continually updating self-experimentation and testing protocols). Therefore, the information presented within has not been approved by an IRB, and use of this information is understood to constitute self-experimentation.

The purpose of this open-source vaccine effort is to reduce risk of harm from SARS-CoV-2, minimally until there is at least one effective commercial vaccine widely available. In addition to providing the results of our own research and experimentation, we hope to motivate others to build on our work, and to pursue diverse evidence-based approaches. Given the immense complexity and variability of individual human biology, it is not possible to predict all potential physiological responses to any vaccine. But as is true for most or all vaccines used on a large scale, there is a tradeoff between a larger known risk from the disease the vaccine is designed to prevent or mitigate, and the smaller risk introduced by the vaccine itself.  If used in enough people, any vaccine poses risks and will cause some degree of harm. Furthermore, certain harm, such as allergic and possibly anaphylactic response, will be readily seen and measured, whereas benefit is more difficult and takes longer to assess. This vaccine is no different; and because quality of delivery is highly dependent on the meticulousness of individual end users, it poses unique risks not posed by typical commercial vaccines.

**Does Not Constitute or Substitute for Medical Advice**

Information presented here is ongoing research, and is not intended as a substitute for medical advice. Radvac is not responsible for the decision to administer, or to receive administration of, any vaccine.

**No Promises or Guarantees of Efficacy**

Vaccines are often received with the false hope of efficacy, without testing to determine the degree of individual immune response. For example, influenza killed about 100,000 people in the U.S. between late 2016 and early 2018. Yet, the influenza vaccines available in that period were substantially less than 50% effective against H3N2, the flu strain mainly responsible for the death toll. Many who died were vaccinated but not protected from the virus, and testing for vaccine-induced immunity was essentially non-existent. Vaccine-induced immunity can be more challenging to assess than immunity due to viral infection, and such is probably the case for the nasal vaccine described here. Because this work is a research undertaking, no expectation is given regarding any degree of efficacy in granting protection against SARS-CoV-2. On the contrary, see possible risks and uncertain benefits below.

**Preventive, not Therapeutic**

Even if this vaccine works as intended, it will not help someone who has been infected. It will only work as a preventive measure taken weeks in advance of virus exposure.

**Not a Clinical Trial**

Any use of the information in this document, or provided in correspondence with us does not constitute 'partnership' or 'recruitment' for an organized trial in any way. The supported conjecture here is given as a starting point for additional individual or organized, sponsored research. As of public release of this information, no organized clinical trials have been performed to test this vaccine.

**Not Approved or Reviewed by the FDA**

The information, procedures, and conclusions presented here have not been approved, or reviewed by the FDA, or any other regulatory body.

**Possible Risks and Uncertain Benefits**

- Immediate allergic or other serious reaction
- Unforeseen long-term effects
- Instillation/administration of the vaccine in an inappropriate way or in an infected area might increase the risk of infection by enhancing viral entry into the body
- Benefits are uncertain. There are extensive published histories of the materials and procedures described in this document, but every novel vaccine should be considered experimental, with the possibility there will be no benefit.

- ○ Even if there are signs of immune response there is no guarantee this response is indicative of protection from SARS-CoV-2 infection, or if protection is achieved, how long it will last.
- ○ Even if the vaccine confers protection from the virus, certain methods for assessing protection--such as measuring antibodies due to previous infection--might not capture vaccine-induced immunity. In such cases, infection, and thus a positive test result, are unlikely to occur. Immunity passports and other privileges given to convalescents might be difficult to obtain for those with vaccine-induced immunity.
- ○ Use of this vaccine may change the efficacy of any future vaccines you may take or that are administered to you, in unknown ways

**No Offer of Service and No Access**

All information in this document or via correspondence is made available as open science. We do not sell equipment or services, e.g. we will not prepare this formulation or any other on behalf of a requestor. All vaccine materials at our disposal are kept in a secured laboratory. We cannot and will not keep materials outside the lab for easy access or distribution. We will not provide laboratory or equipment access for vaccine production.

**Probability and Coincidence**

Vaccines have become associated with negative outcomes that do not result from the vaccine, but that occur immediately following administration. Increasing use of any vaccine will increase the probability of recipients experiencing an unconnected negative outcome. By using the information presented here, you acknowledge the increasing likelihood of such coincidences, and assume full responsibility for any use of the information herein and for real or perceived negative outcomes, irrespective of the cause.

**Self Experimentation**

By utilizing the information and scientific opinions in this document, and in any correspondence with us, you acknowledge and agree that any use to develop and self-administer a substance is an act of self-experimentation. Laws and regulations on such actions may differ in your national or state jurisdiction, and it is your responsibility to be informed about them.

Additionally, the type of work described in this document requires certain equipment and level of skill with laboratory techniques. You agree to assume full responsibility for acquiring proper equipment, knowledge, and training, and for attempting to formulate or administer vaccine.

**License**

All contents of this site are covered by a Creative Commons Attribution 4.0 License (CC BY 4.0). You are free to Share (copy and redistribute the material in any medium or format), and Adapt (fork, alter, and build upon) the material for any purpose. The only requirement is Attribution.

## BACKGROUND

The SARS-CoV-2 virus (a.k.a. 2019-nCoV; disease: COVID-19) is responsible for a worldwide pandemic far beyond the scope of any other public health crisis in living memory. Testing for current infection (molecular testing; RNA; RT- qPCR; LAMP; SHERLOCK; etc.) and testing for prior infection (serological and antibody testing; lateral flow; ELISA; etc.) are complex, and as of late July, 2020, remain unstandardized and unreliable. Some available tests are accurate; however, despite testing company claims of extremely high accuracy, the U.S. CDC and other agencies have warned that virus sampling protocols introduce substantial uncertainty in molecular testing, and that antibody tests are often incorrect [1]. Decisions to resume aspects of normal life, including interpersonal contact, are greatly complicated by such high false positive and negative results, and by relatively long viral incubation (reportedly an average of about 5 days) during most of which a pre-symptomatic person is infectious.

Most leading experts have said reliable testing will be helpful to manage the pandemic and individual exposures, but that the ultimate goal is an effective vaccine.  Unfortunately, a vaccine for the virus is not available outside of small clinical trials, and experts in the U.S. have said commercial vaccines will not be available until early to mid 2021, if all goes well. [2] This document describes the rationale, design, formulation, and intranasal self-administration of a peptide-based vaccine against SARS-CoV-2. Starting in March, 2020, our group has followed these protocols, which are based on prior publications and a rapidly growing preprint literature on SARS-CoV-2, to produce and self-administer multiple generations of incrementally improved vaccines.

There is substantial published information on all aspects of vaccine production, testing, and delivery, some of which is specific to the SARS-CoV-2 virus. Published information is sufficient to create a vaccine and tests for its efficacy on a timeline much shorter than that given for commercial vaccines. Vaccines are the safest of all therapeutic classes. Because of their safety and efficacy, they have by far the highest probability of success in the overall course of clinical trials (over 40%, which is about twice the rate of the next highest class).[3] Because of this and the impact of the pandemic, accelerated commercial trials are planned and have begun that bypass extended animal studies, some of which will proceed straight to human trials.

However, commercial vaccines must be designed to enable large scale production and deployment, and they require regulatory approval for sale, which greatly constrain and slow progress. Critically, certain features of commercial vaccines aren't required for research-level production and testing; therefore, a much shorter timeline is possible for a smaller scale,

---

[1] https://www.cdc.gov/coronavirus/2019-ncov/lab/resources/antibody-tests-guidelines.html
[2] https://www.technologyreview.com/s/615331/a-coronavirus-vaccine-[...]-least-18-monthsif-it-works-at-all/
[3] https://projectalpha.mit.edu/pos/

self-administered vaccine. Herein we describe the formulation of a vaccine using proven, inexpensive, and mostly off-the-shelf components. The open-source SARS-CoV-2 vaccine described can be produced quickly and inexpensively in a wide range of lab and physician office settings. Our group, the Rapid Deployment Vaccine Collaborative (RaDVaC), has produced and first administered the vaccine to ourselves in late April 2020. As of mid August, over 20 of us have self-administered a seventh generation (Gen 7) vaccine. This will be a versioned living document, enabling incremental improvements in vaccine design and testing.

## VACCINE BACKGROUND AND DESIGN (last updated 8/16/20)

There are many advances in vaccine research and technology development that have not appeared in a commercial product, in part because commercial vaccine design and production is constrained by different factors than a small-scale research vaccine. Key attributes for a RaDVaC open-source vaccine are high safety, low cost, and ease of production and administration. Another key feature of the RaDVaC vaccine is rapid iteration of updated designs as new discoveries about the biology and clinical manifestations of SARS-CoV-2 are published in the biomedical literature and on preprint servers. This feature is critical to the RaDVaC mission of rapid development and deployment of effective vaccines. We address all of these in this document, and consider subcategories, such as near-term and long-term safety.

Key issues and some differentiating factors between commercial and research vaccines:

- **Rapidly responsive design.** A key feature of our rapid deployment strategy is the extreme short-term flexibility of design, allowing very fast and efficient updating of vaccine designs in response to recent research. For example, as of mid August, 2020 our most recent vaccine designs incorporate key discoveries about critical determinants of T-cell mediated immunity, which were published in the biomedical literature only weeks before. One important discovery shows that T-cell responses are found in patients almost two decades after recovery from SARS-CoV-1 (a very closely related coronavirus)[4]. The portions of the virus responsible for these very long-lived T-cell responses (CD4 and CD8) are in highly conserved parts of the virus. They are not present in the Spike protein, which is not highly conserved, and which is the primary target of leading vaccines in the race to commercialization. A second publication reveals the identities of 29 experimentally verified, immunodominant CD8 T-cell epitopes of SARS-CoV-2[5]. Some of these epitopes are shared among the small group of long-lived epitopes from SARS-CoV-1, none of which resides in the Spike protein. Only 3 of 29 epitopes lie within the Spike protein, and only one lies within the RBD region of Spike. Another subsequent publication experimentally

---

[4] https://www.nature.com/articles/s41586-020-2550-z
[5] https://www.medrxiv.org/content/10.1101/2020.07.24.20161653v2.article-info

confirmed and extended these findings, also showing that Spike epitopes are not among the top 10 immunodominant CD8 T-cell epitopes[6]. These findings suggest that the Spike protein might not provide extensive and robust T-cell protection, and that RBD will provide even less protection for many people, especially the large numbers of people with MHC haplotypes not represented by these antigens. Again, our latest (Gen 7+) vaccine designs have been updated to include epitopes from these discoveries.

- **Near-term safety.** Near-term safety of the intranasal vaccine described here should be excellent when prepared appropriately. Intranasal vaccines have a long history of demonstrated safety. The vaccine formulations described here can also be used for other delivery routes, including inhalation, oral, or parenteral delivery (injection), but these, especially the latter, should be attempted only by skilled practitioners. In general, a vaccine is more safely used for inhalation into the lungs[7], oral, or intranasal delivery, than for injection. This document is focused on intranasal delivery, but experts will know how to adapt the information herein to another delivery route. For any delivery mode, sourcing of high-quality materials and meticulous preparation are essential for maintaining vaccine safety.

- **Long-term safety.** The long-term safety of any vaccine is currently difficult or impossible to predict. Even widely-deployed commercial vaccines have resulted in serious and unforeseen complications. Vaccines that have shown serious side effects are injectable, whole virus or subunit formulations. There are at least three potentially serious complications that might arise in the long term: tolerance, vaccine-enhanced disease, and adjuvant triggered immune or neurological complications.
  - **Immune tolerance** is a term meaning diminished immunity resulting from exposure to an antigen. This attenuated immune response is commonly observed in food antigens, and in "self" antigens of one's own body. In general, it is thought that extremely large and/or frequent exposures and oral doses lead to tolerance.
  - **Vaccine-enhanced disease**. A small number of injected vaccines have led to enhancement of disease, meaning that infectivity is enhanced, or the disease is made more serious in people who have been vaccinated, relative to unvaccinated controls. This has occurred in response to vaccines for respiratory syncytial virus, Dengue fever, Zika, and SARS. One mechanism is antibody-dependent enhancement (ADE), in which antibodies of the systemic immune system increase immunopathology (in the case of SARS, especially of lung tissue) or otherwise enhance disease. This highlights one important advantage of an intranasal vaccine: a robust mucosal immune response should greatly reduce or prevent this systemic response by abrogating initial infection. One early report on SARS-CoV-2

---

[6] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2.article-info
[7] https://www.sciencedirect.com/science/article/pii/S1549963415000313

suggests that neutralizing antibodies for the SARS-CoV-2 receptor binding domain (RBD) do not exhibit such enhancement.[8],[9] We mention ADE and other possible negative outcomes for a vaccine against SARS-CoV-2 in order to provide sufficient background on risks, but some experts suggest that ADE is not a clear concern in the development of vaccines against this virus.[10]

- ○ **Adjuvant hyperstimulation or toxicity.** Adjuvants help stimulate a robust immune response to a vaccine; however, certain adjuvants have caused hyperstimulation and other serious side effects. For example, alum produces a robust Th2 immune response, but an unbalanced ratio of Th2:Th1. A Th2 polarized response, and alum in particular, have been implicated in immunopathology, including ADE. Adjuvants can also be toxic. As one example, the intranasal use of a detoxified mutant form of *Escherichia coli* Heat Labile Toxin has resulted in transient Bell's palsy, or facial nerve paralysis.[11] One reason for using strong adjuvants in commercial vaccines is to trigger a robust immune response with a single administration, and avoid a booster schedule.

- ● **Stability.** Stability is a key determinant of a commercial vaccine. Formulations that are both safe and effective in a research setting, but with limited shelf life, are generally excluded from commercial products. We have discovered that there are formulations for vaccines that are extremely simple, safe, and effective, but have only short-term stability (on the order of weeks). For example, chitosan gel nanoparticles have been shown to be highly effective and extremely simple to formulate, but short shelf life has contributed to their limited use in commercial vaccines.

- ● **Intranasal delivery.** Intranasal vaccines have advantages over other delivery approaches, including the most common delivery modality, parenteral/injection. Intranasal delivery has been demonstrated to be very safe, with mild side effects typically equal to those seen in placebo-treated subjects.  Importantly, it can elicit not only systemic immunity, but also mucosal immunity at the point of infection for respiratory viruses.[12] Commercial intranasal influenza vaccine is available, and relative to parenteral (injected) administration shows equal efficacy for systemic immunity, but greater efficacy for mucosal immunity at sites of entry (nose, lungs) for respiratory viruses.[13] This is critically important for SARS-CoV-2 because early studies show that a majority of infections begin in the nasal cavity[14]. As of June 2020, most or all commercial SARS-CoV-2 vaccines in development are designed to be injected (parenteral), a route which is unlikely to provide mucosal immunity to infection.

[8] https://www.biorxiv.org/content/10.1101/2020.04.10.036418v1
[9] https://www.nature.com/articles/s41587-020-0577-1
[10] https://www.pnas.org/content/117/15/8218
[11] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2737308/
[12] https://pdfs.semanticscholar.org/6e55/db027b08b367eaac55ec54a730f4c99061ec.pdf
[13] https://www.tandfonline.com/doi/abs/10.1586/erv.12.31
[14] https://www.cell.com/cell/pdf/S0092-8674(20)30675-9.pdf

- **Prime-Boost.** Intranasal delivery can be as effective as injection, but to achieve this level of immune response and protection--especially with milder adjuvants--multiple doses are often required. The initial dose is the prime and subsequent doses are given to boost or increase the immune response. Prior exposure to a pathogen or closely related one has a similar effect to a prime dose of vaccine. The only commonly used intranasal vaccine is for influenza. Since essentially everyone is naturally exposed to influenza, the nasal vaccination with attenuated virus is in essence a booster. Requirement of multiple boosters has limited the commercial production of intranasal (and other mildly to moderately immunostimulating) vaccines for which prior ubiquitous exposure is unlikely.
- **Efficacy**. Intranasal delivery of chitosan-based vaccines have shown mild side effects and high levels of efficacy of both mucosal and systemic immunity, when delivered in a prime-boost regimen (in both animal models and human trials; see one review by Smith et al. [15]).

Given the above key points, it is clear that a rapid-deployment vaccine that is both safe and effective is likely to have multiple attributes that differentiate it from a commercial vaccine. And it becomes clear why the formulation described here has not been used in a commercial product; it is not lack of safety or efficacy, but other factors related to stability, scalability, patient compliance, profitability, and regulatory complexities of commercial vaccines. Commercial vaccines are designed to produce high levels of immunity with a single dose. A prime and multi-boost schedule is used routinely in the research setting, but concerns about compliance (failure to boost) limit commercial deployment.

For multiple reasons, including those given above, intranasal delivery is difficult to achieve for a commercial vaccine; however, for a research vaccine, it is not only relatively easy, in certain cases and for some pathogen classes like respiratory viruses, it is the preferred mode of delivery. Relative to injection, there is no risk of needle injury or transmission of blood-borne infection. Immunization by the intranasal route not only prevents viral infection through the nasal membranes, but also efficiently stimulates a strong mucosal immune response in the lungs and upper respiratory tract. As one example, Gai and colleagues have shown that a SARS vaccine delivered intranasally elicits a robust mucosal immune response, protecting against initial infection, whereas the same vaccine delivered by injection does not.[16] This difference is important because the area of the mucosal surfaces (nasal, lungs, gastrointestinal, urogenital, etc.) is very large, about 200 times the surface area of the skin, and about 70% of pathogens enter through these routes.[17] Compliance is also very high for a single dose because intranasal delivery doesn't involve needles or cause pain. High safety and ease of administration are expected to contribute

---

[15] https://www.tandfonline.com/doi/full/10.4161/hv.27449
[16] https://scholar.google.com/scholar?cluster=1372284195977601349&hl=en&as_sdt=0,22
[17] https://pdfs.semanticscholar.org/6e55/db027b08b367eaac55ec54a730f4c99061ec.pdf

to high rates of immunization. For a recent review of research on nasal nanovaccines, see Bernocchi et al [18] (Table 1).

Synthetic peptide-based vaccines have advantages over the most widely used vaccine designs based on attenuated viruses or even full-length Open Reading Frames (ORFs) of key epitope proteins. Coronavirus Spike and Nucleocapsid full-length proteins have been associated with ADE in animals and human cellular studies. Yasui and colleagues showed that Nucleocapsid vaccination does not provide protective immunity, yet enhances immunopathology.[19] Vaccination with certain epitopes of Spike protein do confer protection, but use of the full length Spike is not recommended. For example, from Tais and colleagues: "… full-length S protein should be used with caution. Kam *et al*. reported that although a recombinant, trimeric SARS-CoV S protein vaccine elicited a protective immune response in mice the anti-S antibodies also mediated antibody-dependent enhancement of viral entry into human B-cells *in vitro*. In another study, ferrets vaccinated with SARS-CoV full-length S protein expressed by a recombinant modified vaccinia Ankara grown in BHK21 and Vero E6 cells show enhanced virulence of hepatitis induced by SARS-CoV. Furthermore, the use of a SARS S protein vaccine may lead to enhanced disease and immunopathology instead of protection as seen for feline coronavirus, feline infectious peritonitis virus. Given these concerns, the use of a SARS vaccine strategy in which the full-length S protein is used may not be optimal for humans. Hence, the best approach would probably be to use small S protein epitopes that are major neutralization determinants."[20] Vaccines against other pathogens that similarly use full-length constructs have also elicited enhancement of viral sensitivity or disease. The vaccine design described here is based on such B-cell peptide epitopes of the S or spike protein, predicted in the literature to be major neutralization determinants, as well as predicted and experimentally tested effective T-cell epitopes. Several are combination B and T-cell epitopes.

Synthetic peptide synthesis provides freedom to design epitopes of sufficient length for immunogenic stimulation, but predicted not to trigger these serious side effects. This approach also allows the use of multiple epitope peptides, either concatemerized in a single, multimeric linear peptide, or as a collection of individual peptides. The approach taken here uses individual B-cell epitope peptides of S protein, and T-cell epitopes from S and possibly other proteins. Certain of the B-cell epitope amino acid sequences contain predicted T-cell epitopes. We have employed a few of these combination epitopes. Later versions of epitopes were selected based on data for neutralizing antibodies and T-cell responses from previously SARS-CoV-2 infected convalescent patients.

---

[18] https://www.ncbi.nlm.nih.gov/pubmed/28698066
[19] https://www.jimmunol.org/content/181/9/6337
[20] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2756483/

Choice of adjuvant is important for safe enhancement of immune response. Many adjuvants have been compared for their ability to elicit various aspects of immune response. These include alum, chitosan, inactive cholera toxin (CT), CpG DNA, monophosphoryl lipid A (MPL), poly (I:C), imiquimod and related compounds, and *E. coli* Heat Labile Toxin. Alum is an aluminum salt (Aluminum potassium sulfate, AlK(SO4)2) and is the most widely used adjuvant in commercial vaccines. It has been used for nearly a century as an effective adjuvant. In direct comparisons, higher doses of alum are superior to most other adjuvants, including in intranasally delivered vaccines. Alum has been found to be equal or superior to most others for eliciting systemic immunity (in IgG1, IgG2a, IgG2b), and superior for eliciting secretory IgA (sIgA) mucosal immunity and protection against virus challenge in animal studies.[21] Poly (I:C) can act as an additional adjuvant; however, it shows weak activity relative to alum, and at higher doses it has triggered severe reactions and autoimmunity in animal models.[22]

Chitosan-based therapeutics have been developed for many biomedical applications. For vaccine applications, chitosan acts as both delivery nanoparticle and adjuvant and has been formulated for intranasal administration, parenteral injection, oral and sublingual delivery, and more. It has been used widely in animal experiments, and it has been used safely in human clinical and preclinical trials (see Smith et al. review of trials by El-Kamary et al. and Atmar et al.[23]) (some trials used the commercial product Viscogel)[24],[25] (also see selected human trials reviewed by Guro Gafvelin and Hans Grönlund, in *Molecular Vaccines: From Prophylaxis to Therapy - Volume 2*, Edited by Matthias Giese; Chapter 39, pp. 624-629;  Springer. See Table 39.1 [26]). The use of chitosan in intranasal delivery of an influenza vaccine to healthy human volunteers produces systemic (IgG) protection, though less than parenteral vaccine with alum adjuvant (although 15 microgram dosage is low)[27]; plus, it also induces mucosal immune response, unlike parenteral vaccine[28]. Chitosan as an adjuvant has been compared to alum and to other adjuvants, alone and as part of an adjuvant cocktail, in injectable and intranasal forms. Chitosan alone is a potent mucosal and systemic adjuvant, and it is synergistic with alum and other adjuvants[29,30]. Importantly, intranasal delivery of chitosan-based vaccines has the ability to elicit both mucosal and systemic immunity.

---

[21] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3868302/
[22] https://www.ncbi.nlm.nih.gov/pubmed/?term=32122615
[23] https://www.tandfonline.com/doi/full/10.4161/hv.27449
[24] https://cordis.europa.eu/project/id/261954/reporting
[25] https://pubmed.ncbi.nlm.nih.gov/25218298/
[26] https://books.google.com/books?id=Oda4BA[...]#v=onepage&q=viscogel%20vaccine&f=false
[27] https://pubmed.ncbi.nlm.nih.gov/15916838/
[28] https://www.sciencedirect.com/science/article/abs/pii/S0169409X01001715
[29] https://pubmed.ncbi.nlm.nih.gov/23933339/
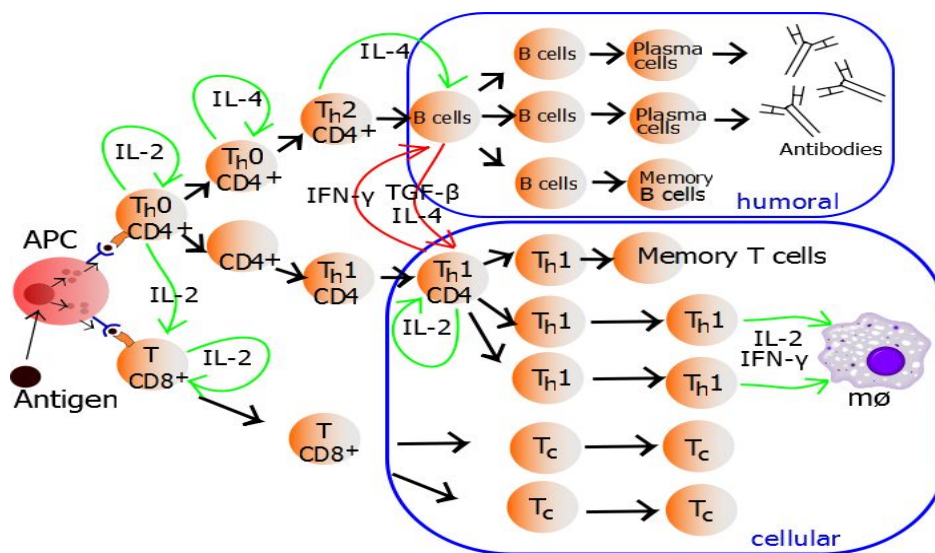[30] https://pubmed.ncbi.nlm.nih.gov/17644205
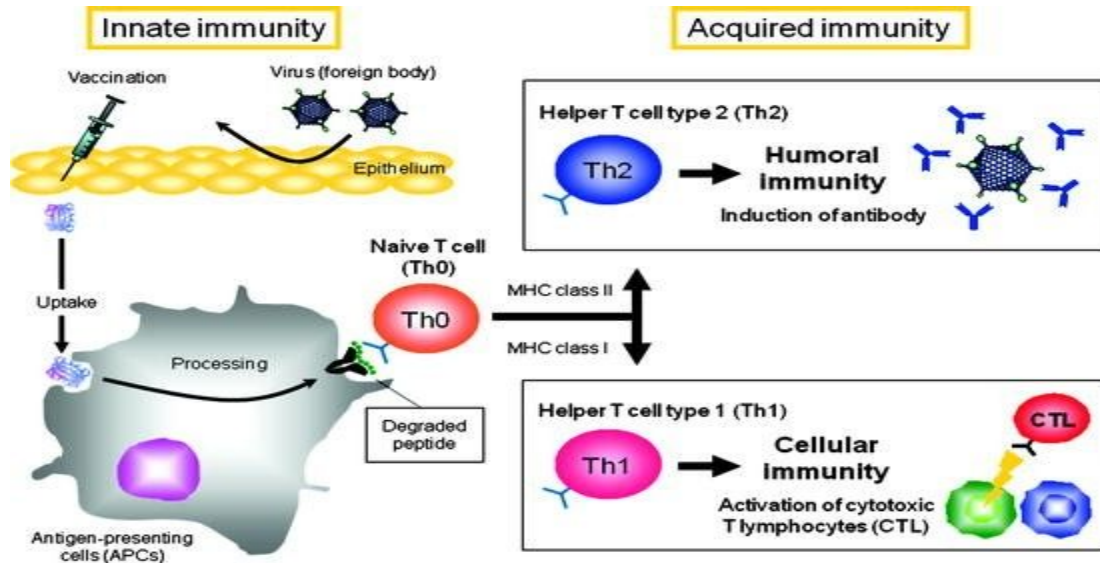
**Schematic showing pathways for Th1 and Th2 acquired immunity (a.k.a. adaptive immunity), and roles of MHC class I and class II.** Figure from Akagi, Baba and Akashi: (https://link.springer.com/chapter/10.1007/12_2011_150).



**Th1/Th2 Model for helper T-cells. An antigen is ingested and processed by an Antigen Presenting Cell (APC). It presents fragments from the antigen to T-cells. The upper, Th0, is a T helper cell. The fragment is presented to it by MHC2. IFN-γ, interferon γ; TGF-β, transforming growth factor β; mø, macrophage; IL-2, interleukin 2; IL-4, interleukin 4. From Wikipedia, https://en.wikipedia.org/wiki/T_helper_cell.**

Chitosan alone elicits both Th1 and Th2 responses, whereas alum triggers strong Th2 and very weak Th1 responses (see Moran et al. for review[31]). In general, the Th1 response plays a dominant role in adaptive immunity (a.k.a. acquired immunity) to viral infections, and is responsible for cellular immunity and killing of infected cells by cytotoxic T lymphocytes (CTL), whereas Th2 is humoral and promotes antibody production. Ideally, both Th1 and Th2 processes are elicited by appropriate antigens and adjuvants, and which to target by vaccination are likely to be pathogen specific. Spike protein stimulation of peripheral blood mononuclear cells (PBMC) of SARS-CoV-2 patients results in secretion of high levels of primarily Th1 cytokines, and elevated but much lower levels of Th2 cytokines [32]. This suggests that SARS-CoV-2 infection provokes a primarily Th1 T-cell response, and a protective vaccine should similarly produce a primarily Th1-based prophylactic response.
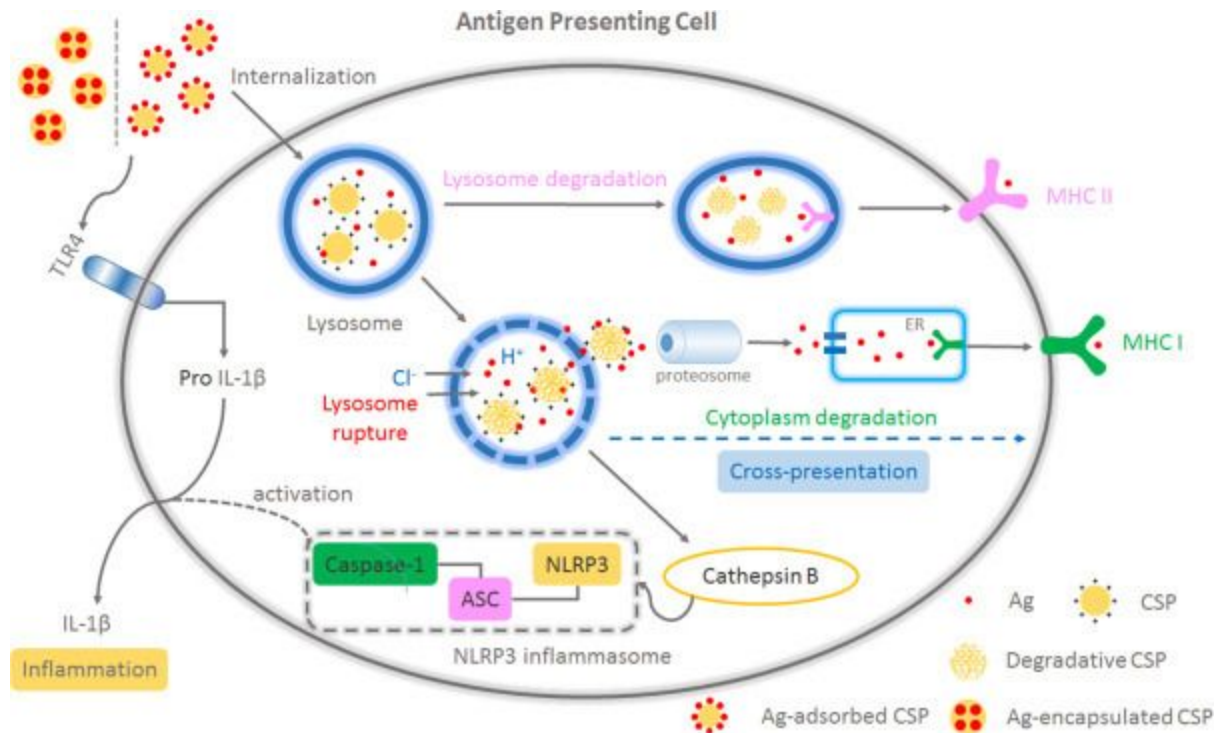
Antibody-dependent enhancement of disease and immunopathology appears to be mediated primarily by a Th2 response [33]. Extensive research on Respiratory Syncytial Virus, SARS, and other respiratory viruses shows that ADE immunopathology will be more likely with the use of alum or other Th2-boosting adjuvant.[34] From Honda-Okubo et al: although an anti-SARS vaccine "formulated with alum protected against mortality, these mice developed severe lung eosinophilia at day 6 postchallenge, reminiscent of the lung pathology induced by alum-adjuvanted RSV vaccines and indicating alum's general lack of suitability as a coronavirus vaccine adjuvant." These concerns make alum alone a poor adjuvant choice, and chitosan the preferred choice. However, seroconversion in response to vaccination becomes more difficult with age; thus, older people might benefit from the addition of additional potent adjuvants, including those that promote some degree of Th2 response. This is an important consideration for SARS-CoV-2, since older people are most susceptible.

[31] https://pubmed.ncbi.nlm.nih.gov/30195140
[32] https://www.medrxiv.org/content/10.1101/2020.04.11.20062349v1
[33] https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035421
[34] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4337527/

**Model of chitosan nanoparticle (CSP)-induced NLRP3 inflammasome activation and cross-presentation: Ag together with CSP (via adsorption or encapsulation) was internalized by APCs. The protonation of the amino groups ("proton sponge effect") leads to an extensive inflow of ions and water into the lysosome, which causes the osmotic swelling and deconstruction of the lysosome. The entrapped components (CSP and Ag) are released and finally presented onto MHC I, by cytoplasm degradation (with proteasome and ER involved); After the rupture, lysosome enzymes, cathepsin B, was also leaked into the cytoplasm, which induced the assembly and activation of NLRP3 complex. The capacity of TLR4 stimulation of CSP also played an important role in the intracellular synthesis of pro IL-1β and triggered the secretion of IL-1β and inflammatory responses, together with NLRP3 activation. (Ag: antigen; CSP: chitosan particle.) Figure and caption from Xia et al., https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7185844/.**

The amount of vaccine delivered is important. Too little antigen is unlikely to elicit a strong immune response, but too much might attenuate response. Mucosal delivery of an antigen has been shown to induce systemic immune tolerance for novel antigens, but probably not for pre-existing antigen exposure. This is the likely explanation of the failure to control autoimmune diseases with mucosal antigen exposure. "Because of pre-existing systemic immunity induced by prior infection or systemic immunization, the likelihood of inducing mucosal tolerance by mucosally administered vaccines is small" and "it is unlikely that mucosal immunization of an individual with even a low pre-existing level of systemic immunity would induce T-cell-mediated systemic

unresponsiveness."[35] Oral delivery might be most important for systemic tolerance. Furthermore, high doses of mucosal antigens appear to be required in humans (50+mg) for inducing systemic tolerance, whereas low doses do not appear to induce systemic tolerance.[36]

One challenge for intranasal vaccines is verifying efficacy. Many published reports show that nasally delivered vaccines have high efficacy for prevention of infection, typically equivalent or superior to injected vaccines for this most important measure. However, efficacy is not as easily measured or predicted by traditional measures, such as anti-virus or anti-epitope antibody titer (e.g. as measured by ELISA) in serum. This is in part because blood is more quantifiable than mucosal secretions, and in part because the primary means of conferring immunity is through mucosal stimulation and response. Measuring efficacy against SARS-CoV-2 is also difficult because the B-cell/antibody response to coronaviruses is highly variable and provides uncertain immune protection. Sustained immune protection appears to be mediated in large part by the T-cell response, which is much more difficult to measure than the antibody response.  In certain cases, nasal vaccination can impart mucosal immunity, but not systemic immunity. In such cases, resistance to viral infection due to mucosal immunity will not be predicted accurately by a negative result for IgG systemic antibody. Antibody can be measured in nasal wash, however this is less quantitative and reliable than measurement in serum. Because of such issues, alternative approaches to predicting overall immunity have been developed and published. These include systems biology approaches of blood-based profiling of immune proteins and transcriptomics.

**Summary of key general features of a citizen-science vaccine:**

- **Safe ingredients.** Long history of published results.
- **Ease and robustness of production.** Simple to make but with the possible tradeoff of short stability. Chitosan nanoparticles form spontaneously and reproducibly, over a wide range of conditions and ingredient concentrations.
- **Intranasal delivery.** The preferred delivery mode for respiratory and nasal viruses. Highest safety profile. Potentially stimulates both protective mucosal immunity at the site of infection, and systemic immunity.
- **Peptide epitope antigens.** Short peptides of linear epitopes are easiest to procure or produce, and are predicted to function best without specific structural constraints. These can be mined from a rich literature, or produced de novo. Antigens for incorporation into a vaccine can be produced synthetically or as recombinant expression proteins.
- **Booster schedule.** Allows use of intranasal delivery and a lower dose or milder adjuvant, yet has the potential to produce immune responses equivalent to a single dose of hyper-stimulatory adjuvants.

---

[35] https://www.jimmunol.org/content/179/9/5633.full
[36] https://academic.oup.com/intimm/article/26/9/517/2950826

## KEY TECHNICAL FEATURES AND SPECIFICATIONS

Certain technical features of the RaDVaC strategy are key to a citizen-science vaccine.

1. **Intranasal delivery** is probably the safest choice of administration routes. For a respiratory virus, intranasal delivery has the advantage of eliciting a mucosal immune response at sites of viral entry. Intranasal delivery can also elicit systemic immunity, although often not as robust as parenteral delivery after a single dose.

2. **Chitosan nanoparticle and adjuvant(s**). Successful vaccines depend on successful delivery and on immunostimulatory adjuvants. Many adjuvants are proven and available, with various strengths and weaknesses. A leading nanoparticle combined nasal delivery and adjuvant system is based on chitosan and sodium triphosphate (STP) a.k.a. tripolyphosphate (TPP)[37], which is the present top choice for this intranasal vaccine. Chitosan is a partly or completely deacetylated derivative of chitin, the linear polysaccharide found in the shells of crustaceans like shrimp. Nanoparticles of various sizes can be created with simple tunable parameters.[38],[39] The virus has an average size of about 100 nm to 150 nm.[40] Nanoparticle size ideally will be tuned to be between 100 nm and 200 nm. As described above, chitosan has been shown to be safe and well tolerated, and intranasal delivery elicits both mucosal and systemic immune responses.
   a. Possible additional adjuvants/immunostimulants. Chitosan is a Th1/Th2 immune triggering, self-adjuvanting polysaccharide; therefore, additional adjuvants or immunostimulants are not strictly necessary, and none will be incorporated into the initial formulation. However, if chitosan alone produces insufficient immune stimulation, we are considering experimenting with the incorporation of other adjuvants that enhance a Th1 or specific T-cell targeted response. Good candidates are alum (Th2 biased), imiquimod (TLR7 agonist, Th1 biased), all-trans retinoic acid (ATRA)[41], poly (I:C) derivatives (TLR3 agonist, Th1 biased), compound 48/80 (mast cell agonist with balanced Th1/Th2/Th17 response)[42], and other TLR[43] (mostly Th1 biased) and mast cell[44] agonists .

3. **Peptide antigen.** A peptide-based vaccine is the method of choice.

---

[37] https://www.frontiersin.org/articles/10.3389/fimmu.2018.00934/full
[38] https://www.nature.com/articles/s41598-018-23064-4
[39] https://www.ncbi.nlm.nih.gov/pubmed/28578269
[40] https://www.nejm.org/doi/full/10.1056/NEJMoa2001017
[41] https://jvi.asm.org/content/85/16/8316
[42] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2743390/
[43] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3000864/
[44] https://www.nature.com/articles/nm1757

    a. Multimeric/multi-epitope vaccines have been shown to be effective. The approach taken here uses multiple peptides, each of which will carry one or more B and/or T-cell epitopes.

    b. Synthetic peptides are inexpensive, and can be made to order quickly by many commercial peptide producers. They can be synthesized with many possible chemical modifications that have been reported to increase immunogenicity.

    c. Early versions of the RaDVaC vaccine contained simple linear epitopes, with no special regard to conformation. Some of the B-cell epitope peptides from Gen 3 onward were conformationally constrained with disulfide bonds.  Ideally, 3D structures of viral proteins should be imaged and linear epitopes should be chosen that either don't require special conformational constraints, or attempts should be made to achieve natural structural conformation of the epitope peptide.

    d. Other antigens are possible and can be delivered by Chitosan intranasally, including DNA or RNA.

4. **Epitope selection** is critical. B-cell and T-cell epitopes have been selected and published by others. Multiple epitopes of both types should be selected, preferably in highly conserved regions of the virus. This gives a higher probability that one epitope will successfully stimulate immunity, rather than relying on a single epitope.

    a. B-cell epitopes. Studies of antibody mapping from convalescent patients help identify portions of the virus available for antibody binding to B-cell epitopes, and some of these antibodies have been shown to neutralize viral infection in human cellular studies. Certain B-cell epitopes that score highly by common machine learning predictions have not been reported at high frequency in convalescent sera. This might be due to linear peptide based approaches used to map epitopes, the extensive glycan shield around the virus, or other complicating factors. Whatever the explanation, we regard the convalescent antibody data as superior to purely computational predictions.

    b. T-cell epitopes. Empirical methods similar to but more complex than those used for B-cell epitope selection have been used for selecting superior T-cell epitopes.

5. **Testing**. Success of mucosal and systemic immune stimulation will be assessed by testing antibody titers of nasal wash, saliva, and serum. Testing of immunity will be performed using standard assays and reagents that are currently in development, as well as the use of newer technologies, such as transcriptome profiling of peripheral blood mononuclear cells (PBMCs).

## GOALS

1. Design and incrementally improve a vaccine made with commercially available materials, and with the following properties:

a. All ingredients have undergone extensive testing for efficacy and safety in animal models, and preferably in human trials. Delivery and adjuvant materials and technologies must have decades of proven safety.

b. Use of best practices to achieve high probability of efficacy.

c. Mode of administration must be safe and proven. Intranasal delivery is preferred for safety and for establishment of both mucosal and systemic immunity. Inhalation and injection are possible delivery modes for this vaccine but should only be pursued by those with sufficient training and experience.

d. Vaccine should be extremely easy to make (including sourcing of ingredients) and administer.

2. Produce vaccine for initial testing, and self-administration.

3. Produce and administer updated generations of vaccines as new information becomes available.

4. Design and implement testing for immune responses to the vaccine. Tests will be performed with published methods, and new methods should be developed to predict efficacy. Ideally, biological samples such as blood and nasal wash are collected before initial vaccination, and at various time points.

5. Disseminate information enabling many others to produce the vaccine. The only custom components are the epitope peptides. By themselves, these synthetic peptides are not drugs, and might be distributed to facilitate production and use.

6. Maintain ongoing long-term self-monitoring of health and symptoms to collect empirical evidence of degree of efficacy and possible unforeseen effects.

7. Create an online forum for the exchange of data, ideas and best practices with other individuals and groups. Over time, results can be aggregated for broader analysis.

## MATERIALS AND METHODS

### Selection and purchase of the vaccine ingredients

All materials and ingredients are commercially available. However, some vendor deliveries might be interrupted by public health measures. The vaccine is very simple and consists of five ingredients: epitope peptide(s), chitosan, sodium triphosphate, water, and sodium chloride.

- Epitope peptides. Multiple vendor options are available for synthetic peptides. Peptides or concatemers of the selected epitopes can also be produced by other means, such as recombinant plasmid-based expression and purification (e.g. E. coli His tag). Options should be considered and tested, as supply channels might be disrupted by the pandemic.

- Chitosan. Multiple molecular weights and suppliers have been tested and published. Acceptable ranges: medium molecular weight chitosan, range of 190-310 kDa. Deacetylation = 75% to 90%.
- Sodium triphosphate, $Na_5P_3O_{10}$. Multiple suppliers.
- Sodium chloride (NaCl). 5M stock solution.
- $dH_2O$, deionized water (widely available)
- OPTIONAL: Base ($NH_4OH$, or NaOH) and/or acid (acetic, $CH_3COOH$); (only for low solubility acidic peptides)

## Preferred equipment and materials

Certain equipment is required for efficient vaccine production and administration. Vaccine can be produced without specialized laboratory equipment but the process is more laborious and the results are likely to be more variable.

- Pipettes: 1000 microliter, 200 microliter
- Sterile filtered pipette tips: 1000 microliter, 200 microliter
- Scale: gram scale accurate to 0.1 grams, or jeweler's scale for very small amounts
- Clean spatula for dry reagents
- Small beakers for mixing and stirring. 10 ml to 25 ml beaker for stirring
- Small bottles for stock solutions; 100 ml to 250 ml
- 15 ml conical tubes
- 50 ml conical tubes
- 1.5 to 2 ml microcentrifuge tubes
- Stirring apparatus: magnetic stir plate and small stir bar to fit in beaker
- OPTIONAL: pH strips, range at least 5.0 to 9.0
- Nasal spray apparatus. These are available through multiple vendors. We selected small nasal spray bottles (about 5 milliliter) with a pump-top apparatus. A tube stem about 4 cm long and approximately 1 mm inner diameter extends from the bottom of the apparatus, which can be placed into the bottom of a 2-ml tube containing vaccine. Our spray apparatus delivers about 100 microliters per pump.

## Selection of epitopes

Epitopes are a critical element of a vaccine because they are specific to each pathogen. An epitope is the portion of an antigen (typically a protein) that interacts with the immune system, such as through binding of antibodies or T-cell receptors. Multiple data types can be used in the selection of epitopes. This section describes general guidelines and issues for consideration in epitope selection and design.

T-cells are critically important determinants of immunity to pathogens, and early research has shown this to be the case for SARS-CoV-2.

- **B-cell and T-cell immunodominance of SARS-CoV-2**. Immunodominance is a phenomenon that means a fairly small fraction of possible epitopes are responsible for a majority of immunity, and immunodominance has been reported for both B-cell and T-cell immune responses to SARS-CoV-2. These findings are supportive of the peptide vaccine approach, since only a limited portion of the virus needs to be represented in the vaccine for it to be effective.
    - Several publications show that about half a dozen epitopes are responsible for the majority of antibody binding to the Spike protein, most of which are neutralizing antibodies.
    - A publication by Snyder and colleagues shows a few epitope peptides are responsible for over 50% of of CD8 T-cell receptor binding events[45].
- **The critical importance of T-cell epitopes and immunity.** Antibodies get a lot of press attention (in large part because simple commercial antibody tests are widely available but T-cell tests are more complex and must be performed on isolated cells, and therefore are done in specialized research labs) but the most protective immune response consists of robust humoral (B-cell mediated) and cellular (T-cell mediated) responses. Early indications suggest T-cell responses might be key to both minimizing severity of illness and to longevity of immunity. Here are a few key findings about the importance of T-cell immunity to SARS-CoV-2 infection:
    - Mild COVID-19 is seen in convalescents with robust T-cell responses, including those who do not have detectable antibodies (are seronegative)[46]
    - T-cell exhaustion or deficiency is correlated with disease severity[47,48,49]
    - Population MHC class I binding is predictive of mortality[50].
    - Vaccine-enhanced disease involves a Th2-polarized response that is CD8 T-cell deficient[51]. A robust CD8 response is observed in people and animal models who do not develop vaccine-enhanced disease.
    - As of late July 2020, multiple reports have shown rapid decline of anti-SARS-CoV-2 antibodies in convalescents. It remains unclear how this decline impacts protection from reinfection or severity of disease, but initial results also show that T-cell immunity might be very long-lived. One report by Le Bert and

---

[45] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2
[46] https://www.biorxiv.org/content/10.1101/2020.06.29.174888v1
[47] https://www.nature.com/articles/s41423-020-0401-3
[48] https://www.frontiersin.org/articles/10.3389/fimmu.2020.00827/full
[49] https://www.medrxiv.org/content/10.1101/2020.06.16.20130914v1
[50] https://www.medrxiv.org/content/10.1101/2020.05.08.20095430v3.full
[51] https://link.springer.com/content/pdf/10.1007/s12026-007-0071-6.pdf

colleagues shows that patients who recovered in 2003 from SARS-CoV-1 infection harbor SARS-CoV-2 cross-reactive T-cells 17 years later[52].

- Optimal T-cell epitopes vary by ethnicity. Coverage of the vast majority of human MHC haplotypes (MHC in humans is also called Human Leukocyte Antigen, or HLA) has been reported for many pathogens, including SARS-CoV-2 (for example, as published by Liu and colleagues[53]). Such publications are useful for selection of T-cell epitope peptides for use in specific geographical regions and ethnicities. However, only one of the long-lived T-cell epitopes described by Le Bert et al is present in this set of predicted epitopes, and it is predicted by Liu et al to be optimal for MHC1 and MHC2 haplotypes found mainly in Central Africa, a population not represented in the Singapore convalescents (mainly East Asian and European ancestry). And the top immunodominant MHC Class I / CD8 epitopes empirically verified in the T-cells of convalescents[54] are not represented in the computationally predicted set of Liu. Therefore, such computational predictions should be used with the caveat that they are not always consistent with empirical data.

- **Peptide epitopes** are preferred because they have the following advantages over other epitopes:
  - Well defined
  - Potentially pure
  - Enrichment of antigenic targets; not accompanied by substantial unnecessary and potentially counterproductive/non-neutralizing antigenic targets. Some critical neutralizing epitopes can be very small, less than 20 amino acids.
  - Short production/turnaround time
  - Scalable; we currently use 50 to 100 micrograms total peptide per dose
  - Inexpensive relative to other epitope/antigen choices

- **Empirical evidence** should dominate selection criteria. Here are some best types of evidence:
  - Mapping of epitopes in blood and other samples collected from convalescent patients (ideally stratified by severity of illness). This can be accomplished by a few primary means:
    - 3D structural studies and modeling of neutralizing antibody binding to a viral antigen (e.g. Spike protein)
    - Mapping of linear B-cell epitopes by binding antibodies in convalescent sera to a library of peptides representing viral antigens

---

[52] https://pubmed.ncbi.nlm.nih.gov/32668444/
[53] https://www.cell.com/cell-systems/fulltext/S2405-4712(20)30238-6
[54] https://www.medrxiv.org/content/10.1101/2020.07.31.20165647v2

- ■ Mapping of T-cell epitopes by stimulating convalescent T-cells with epitope peptides, and measuring their response (e.g. cytokine secretion; ELISpot)
  - ○ Epitope peptides from a peptide vaccine that has shown protection against infection
  - ○ Successful use of epitope peptides in vaccines that elicit antibodies (or serum) effective in virus neutralization assays. B-cell epitopes that allow antibody binding to the virus but don't block viral function might increase risk of antibody-dependent enhancement.
  - ○ Mapped epitopes that are effective in virus neutralization assays (e.g. peptides compete with viral sequences in cellular infection assays).
  - ○ Successful use of epitope peptides in vaccines that elicit T-cell responses, or peptides shown to stimulate T-cells or cytokine production in ELISpot or other T-cell assay in cells from convalescents.
- **Multiple epitopes with low mutant escape potential.** It is important to note that most published neutralizing antibodies target Spike RBD, as do many vaccines in commercial development. However, given the high degree of mutability of the RBD portion of Spike, it is highly recommended to identify and select targets outside the RBD because of mutant escape potential. This strategy is summarized by Brouwer and colleagues: "In light of the rapid emergence of escape mutants in the RBD of SARS-CoV-1 and MERS, monoclonal NAbs targeting other epitopes than the RBD are a valuable component of any therapeutic antibody cocktail. Indeed, therapeutic antibody cocktails with a variety of specificities have been used successfully against Ebola virus disease and are being tested widely in clinical trials for HIV-1. NAbs targeting non-RBD epitopes have been identified for SARS-CoV-1 and MERS, supporting the rationale..." for SARS-CoV-2[55]. Three neutralizing epitopes described in the following section, containing or immediately adjacent to the Spike protease recognition sites and heptad repeat 2 (HR2) fusogenic domain are highly conserved among these 3 coronaviruses, suggesting that they have very low mutant escape potential.
- **Immunodominance and limited effective epitope space**. Immunodominance is the phenomenon of a majority of antibody and/or T-cell responses to a small number of epitopes, despite a large number of possible epitopes within the virus. One early but thorough preprint by Zhang and colleagues suggests immunodominance is a feature of the immune response to SARS-CoV-2[56]. Farrera and colleagues reported an overlapping set of immunodominant B-cell epitopes in a study of Swiss convalescent patients[57]. Most of these were subsequently confirmed by Li and colleagues in the first large-scale linear

---

[55] https://science.sciencemag.org/content/early/2020/06/15/science.abc5902
[56] https://www.biorxiv.org/content/10.1101/2020.04.23.056853v2.article-info
[57] https://www.medrxiv.org/content/10.1101/2020.06.15.20131391v1

B-cell epitope mapping of over 1,000 convalescent patients in Wuhan, China[58]. The emerging consensus suggests that there are about half a dozen dominant B-cell epitopes, which are the same in European and Chinese patients. The majority are found in key functional portions of the Spike protein, including the following (amino acid coordinates shown are approximate):

- Spike 450-500; ACE2 binding residues of the RBD (Zhang et al); low degree of conservation; probably moderate to high mutant escape potential
- Spike 655-685; immediately adjacent to the S1/S2 protease cleavage sites; high degree of conservation in the antibody-binding N terminal portion of the epitope; likely low mutant escape potential.
- Spike 805-820; spanning the S2' protease cleavage site (815-816); high degree of conservation; likely low mutant escape potential.
- Spike 1145-1160; adjacent to the heptad repeat 2 (HR2) region, which is critical for fusion of the viral and host cell membranes of the SARS virus[59]; very high degree of conservation; likely extremely low mutant escape potential.

- **3D modeling of viral antigens** (especially Spike protein) is very helpful in B-cell epitope selection and peptide design.
  - Surface loop structures are often good antibody binding sites. Unstructured, hydrophilic loops that are stabilized by a minimal number of neighboring amino acid residues make good linear (rather than conformational) epitopes. One prime example in SARS-CoV-2 is Spike 804 to 818, the TMPRSS2 cleavage site (one of a few immunodominant epitopes reported by both Farrera et al and Li et al).
  - Certain amino acid residues are overrepresented and underrepresented in preferred antigens. According to structural studies, these amino acids are overrepresented (from most overrepresented): W, Q, P, K, S, E, N, H, C. And these are underrepresented (from most underrepresented): A, V, I, F, L, Y [60].
  - Linear epitopes (single peptide chain of nearby amino acids) are easier to map than structural or conformational epitopes (amino acids that are either on separate proteins, or are far apart on a protein sequence but in close proximity in the folded protein). However, conformational epitopes can be critically important and can be designed as single peptides that join discontinuous portions of virus proteins, or can be produced as multiple peptides that are predicted to physically interact into relatively stable conformations.
  - There are multiple excellent 3D modeling packages and online servers. The NCBI supported iCn3d is very good and allows automatic access to Protein Database (PDB) files of viral protein structures.

---

[58] https://www.medrxiv.org/content/10.1101/2020.07.13.20152587v1.full.pdf
[59] https://www.thelancet.com/journals/lancet/article/PIIS014067360415788-7/fulltext
[60] https://www.jimmunol.org/content/181/9/6230.long

https://www.ncbi.nlm.nih.gov/Structure/icn3d/full.html. Multiple 3D models of SARS-CoV-2 proteins are available, including Spike in various conformations, and complexed with antibodies and with the ACE2 receptor.

- **Physical properties of peptides.** Certain peptides have excellent physical properties such as solubility and stability against proteolysis, and are synthesized easily; others are more problematic. Peptides with marginal properties can be improved by amino acid substitutions, additions, or subtractions. Bear in mind that changes to the viral sequence might substantially alter antigen recognition. It is likely preferable to include peptides that are in critical functional regions of the virus, with minor modifications to improve their use in a vaccine, than to exclude them. It is widely reported throughout the immunology and vaccine literature that both T and B-cell immunity are retained to varying degrees for new mutant strains. It has been reported that T-cell responses to SARS-CoV-2 are present in 34% to 60% of people not exposed to SARS-CoV-2, and are likely due to exposures to distantly related coronaviruses (most epitopes less than 50% aa identity)[61], [62]. Therefore, conservatively modified epitope peptides should be expected to provide high levels of protection, although possibly not equivalent to the unaltered viral amino acid sequences.
  - **Length.** Binding of the S protein to the cellular receptor ACE2 is mediated through the receptor binding domain (RBD). The RBD has been mapped for the related SARS-CoV-1 virus, and for SARS-CoV-2. ACE2 binding residues in RBD have been published widely and can be visualized in Figure 3 of Tarek et al[63]. However, multiple studies using short linear peptides for mapping epitopes bound by antibodies in convalescent sera reveal that only low signals are found in peptides within the RBD. This contrasts with several publications showing the isolation of neutralizing antibodies that bind to the RBD. Most of these mapping studies use epitope peptides of 12 to 15 amino acids, while Zhang et al use longer peptides of 20 to 25 amino acids and show that antibodies in convalescent sera show at least moderate binding to multiple RBD epitopes. Therefore, the peptides we have used in our vaccine formulations, especially in the RBD are longer than 20 amino acids, and as long as 40 amino acids.
  - **Solubility.** Certain peptides of the high-scoring epitopes are predicted to have low solubility in water. This complicates formulation, since organic solvent or pH adjustment is required to solubilize. When choosing epitopes and peptides, bear in mind aqueous solubility. Solubility calculators are helpful in this process (for example, see [64] and [65]). Marginal solubility of peptides can be improved by amino

---

[61] https://www.cell.com/cell/fulltext/S0092-8674(20)30610-3#articleInformation
[62] https://www.medrxiv.org/content/10.1101/2020.04.17.20061440v1
[63] https://www.medrxiv.org/content/10.1101/2020.04.25.20079426v1
[64] https://pepcalc.com/peptide-solubility-calculator.php
[65] https://www.thermofisher.com/us/en/home/life-science/[...]/peptide-analyzing-tool.html

acid substitutions, additions, or subtractions. Acidic (Asp, Glu) and basic (Arg, His, Lys) amino acids aid solubility; combinations of acidic plus basic amino acids can keep the overall peptide charge near neutral.

- ○ **Stabilization**. Circular peptides are more stable. Synthesis options are available to produce N-terminal to C-terminal cyclization, or to place cysteines in strategic positions on epitope peptides for disulfide formation. Cyclization stabilizes 3D conformation, and increases resistance to exopeptidase degradation. Disulfide formation is quick, easy, and inexpensive. Cysteines should be designed into epitope peptides at positions that do not change or interfere with normal epitope function, or do so as little as possible. Blocking the N terminus of a peptide also has been reported to stabilize it against proteolysis. [66] Peptides might be either N-terminal acetylated or biotinylated.

- ● **Epitopes for B-cell and T-cell responses can be quite different**, and there are critical differentiating features. Here are key considerations in the selection and design of B-cell and T-cell epitopes.
    - ○ T-cell epitopes are presented as short linear peptide antigens, but B-cell epitopes (which can be linear or conformational) are presented in their native conformation.
    - ○ Linear B-cell epitope selection is typically easier to base on empirical data than T-cell epitope selection. The B-cell response is relatively easily measured by antibodies, but the T-cell response is more difficult to measure and more rarely performed. Antibody testing is a large-scale commercial activity (using lateral flow, ELISA, etc.) but T-cell testing is generally a research activity (using ELISpot, etc.). Therefore, T-cell epitopes are often based on computational prediction.
    - ○ There are 2 classes of T-cell epitopes: MHC Class I and MHC Class II (for a thorough review, see Rock et al.[67]). Core motif binding of both Class I and Class II is about 9 amino acids but both are variable. The Class I binding site has closed ends, and epitopes range from 8 to 15 aa, but infrequently diverge from 9 aa. Class II binding is open-ended, and natural epitopes extend longer than the 9 aa core, and range from 13 to 25 aa[68]. Many computational predictive algorithms are available, and a large number of preprints provide Class I and Class II predictions for inclusion in a vaccine. If empirical data are available for protective T-cell epitopes (e.g. Le Bert et al and Zhang et al[69]), these data should guide T-cell epitope selection.
    - ○ Immune responses (both B and T-cell) can vary substantially with different ancestry/ethnicity. We have used predictions and empirical data for wide

---

[66] https://www.pnas.org/content/pnas/102/2/413.full.pdf
[67] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5159193/
[68] https://pubmed.ncbi.nlm.nih.gov/1380674/
[69] https://pubmed.ncbi.nlm.nih.gov/32668444/

coverage of immune responses (our group is highly ethnically diverse). For specific geographies and ancestries, local immune response information should be taken into account.

○ Although there can be differences between T and B-cell epitopes, they can overlap. If possible, peptides that contain experimentally validated epitopes for both B and T-cells are ideal.

We have produced a few iterations of vaccine, and for each we selected multiple B-cell and T-cell epitopes. Which B-cell epitopes elicit antibody responses can be verified by immunoassay testing, and these epitope peptides can be carried forward in updated versions of vaccines. The use of multiple epitopes also ensures that, if vaccination is successful in eliciting immunity against, say, 2 or 3 epitopes, then a single mutation will not allow the virus to evade immunity. Mutating virus and epitope variants should be monitored (nextstrain.org, bioRxiv, etc) , and new epitope peptides incorporated into evolving designs as needed.[70]

Mapping of antibodies from people previously infected but recovered (convalescent) have shown that there are key regions for antibody binding. SARS and MERS are lethal coronaviruses that have been used for comparison and cross-reactivity of convalescent antibodies. Jiang et al used SARS-CoV-2 specific proteome microarrays to show that S and N proteins bind both IgG and IgM, and the C terminal portion of the N protein exhibits the highest convalescent serum antibody binding signal of all tested proteins.[71] Antibodies from convalescent patients have been mapped to short, linear epitope peptides by Poh and colleagues[72] (S protein only), Wang et al [73], and the previously mentioned Zhang et al, Farrera et al, and Li et al.

Neutralizing (or protective) antibodies are preferred to guide epitope selection. Antibodies to certain functional regions of the virus will neutralize its activity and infectivity. Many antibodies bind to the virus and some will inhibit function but only a subset of all antibodies will truly neutralize the ability of the virus to infecT-cells and replicate. Ongoing research is helping to identify which portions of the virus are key to neutralizing activity. Neutralizing activity is not observed in antibodies associated with antibody-dependent enhancement of disease (ADE).

High-resolution linear epitope mapping by Wang et al was done with arrays of peptides covering the entire length of ten SARS-CoV-2 proteins. Length of peptides is 15aa, and peptides overlap by 5aa. Convalescent sera IgG and IgM were applied separately to the arrays. Binding events to a given peptide but not to adjacent peptides in the array indicate tight binding primarily to the central 5aa portion. Table 3 shows the 61 bound epitopes. Only five of these span more than one peptide. One in the S protein spans 15aa or three peptides (start = 806). These stretches are

---

[70] https://nextstrain.org/ncov
[71] https://www.medrxiv.org/content/10.1101/2020.03.20.20039495v1
[72] https://www.biorxiv.org/content/10.1101/2020.03.30.015461v1
[73] https://www.biorxiv.org/content/10.1101/2020.03.26.994756v1

highly suggestive of a long linear epitope, whereas shorter epitopes are potentially a portion of a structural epitope. Moreover, the longer sequence provides a larger target. The longer S protein epitope binds both IgG and IgM, and a largely overlapping epitope binds convalescent antibodies in the study by Poh and colleagues, and produces the highest signal in the later and larger-scale linear epitope mapping study by Li and colleagues. The epitope at this position (about Spike 805-820) spans a key proteolytic cleavage site required for viral entry into host cells. We included this epitope in Gen 3+ vaccine formulas, and as updated and progressively rich scientific information has become publicly available, we have added additional B-cell and T-cell epitopes to successive generations of vaccine. Each of these additions is detailed with references in the sections below.

## Peptides and vaccine composition

In order for the vaccine to be produced relatively easily, peptides must be water soluble. Through experimentation with various peptides, and addition of soluble residues or substitution of hydrophobic residues with soluble ones, we are able to achieve solubility in water while retaining conservative positions of top scoring epitopes represented on these peptides. We have reversed prior substitutions and propose additional reversals if solubility can be achieved through alternate means. Amino acid substitutions are shown as lower-case letters, and conservative substitutions are highlighted in green, while non-conservative substitutions are highlighted in red. Previously tested and retired (due to low solubility) or conditional or prospective peptides are listed in Supplemental Materials. Peptide synthesis is most efficient if certain guidelines are followed (see for example[74]).

3D structural models of a few of the B-cell, protein surface-exposed peptides are shown in figures below. For these we used the imaging website iCn3d[75]. Loop amino acid residues of each epitope are highlighted in yellow in selected images. We are testing disulfide circularized peptides to promote stability and to approximate natural conformation. These peptide names end in cir, e.g. "Spike 804-820cir". Most of these peptides were circularized by disulfide formation, with exceptions noted below. We did not order these synthesized with disulfide bridge, but performed disulfide formation ourselves by a simple incubation with charcoal as previously described[76].

Certain portions of viral sequence are represented in more than one peptide. There are multiple reasons for why we employed this strategy. In one case (Spike 462-501) was difficult to synthesize, and was replaced by two shorter peptides (Spike 462-476 and Spike 478-502) with

---

[74] https://www.thermofisher.com/us/en/home/life-science[...]pierce-protein-methods/peptide-design.html
[75] https://www.ncbi.nlm.nih.gov/Structure/icn3d/full.html
[76] https://pubmed.ncbi.nlm.nih.gov/9606016/

slightly altered sequences to ensure higher amino acid coupling efficiencies. These shorter peptides were included in the Gen 5 vaccine. Once Spike 462-501 was successfully synthesized, it was added to the Gen 6 vaccine, in addition to Spike 462-476 and Spike 478-502. In another case, Spike 804-820cir was synthesized first and used in early generations of vaccine, and then Spike 802-823cir was synthesized to add potentially important flanking sequences to this protease recognition loop sequence on the surface of the Spike protein.

Additional details can be found below on each epitope peptide, but for convenience, the most recent vaccine peptide compositions are listed here. Complete epitope peptide sequences are shown; an asterisk at the end of a sequence means a peptide has been circularized by disulfide bond formation.

**Gen 7 vaccine**; Same peptide composition as Gen 6 vaccine except with the addition of three Spike B-cell peptides, and two Nucleocapsid protein T-cell (CD4 and CD8) peptides. Starting Aug 4, 2020, many people have self-administered vaccine containing the peptides listed below, at about 5 micrograms of each peptide per dose of vaccine.

1. **Spike 436-460, a.k.a. Spike1,** NSNNLDSKVGGNYNYLYRLFRKSN
2. **Spike 462-476,** KPFERDISTEIYQAd
3. **Spike 478-502,** kPCNGVEGFNCYFPLQSYGhQPTNG*
4. **Spike 550-574cir,** cgLTESNKKFLPFQQgGRDIADTcD*
5. **Spike 375cir,** cSrdYNSASFSTFKsYGVSPTKcND*
6. **Spike 522cir,** CGPKKSTNLVKNKsVNFNFNcd*
7. **Spike 804-820cir,** cILPDPSKPSKRSFcgD*
8. **Spike 802-823cir,** FSQcLPDPSKPSKRSFcEDLLF*
9. **Orf1ab 1544-1564cir,** cFHLDGEVITFDNLKTLLSLREct
10. **Spike 462-501,** KPeERDgSTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTN (being considered for retirement in Gen 8+ due to very inefficient synthesis and concerns about conformational correctness)
11. **Spike 21,** RTQLPPAYTNSFTRGVYYPDK
12. **Spike 660,** YECDIPIGAGICASYQTQTNSPRRA*
13. **Spike 1145,** LDSFKEELDKYFKNHTSP
14. **Nuc 100-120,** KMKDLSPRWYFYYLGTGPEAG
15. **Nuc 321-345,** GMEVTPSGTWLTYTGAIKLDDKDPN

**Gen 6 vaccine**; Same peptide composition as Gen 5 vaccine except with the addition of Spike 462-501. Starting June 8, 2020, many people have self-administered vaccine containing the peptides listed below, at about 5 to 7 micrograms of each peptide per dose of vaccine.

1. **Spike 436-460, a.k.a. Spike1,** NSNNLDSKVGGNYNYLYRLFRKSN

2. **Spike 462-476,** KPFERDISTEIYQAd
3. **Spike 478-502,** kPCNGVEGFNCYFPLQSYGhQPTNG*
4. **Spike 550-574cir,** cgLTESNKKFLPFQQgGRDIADTcD*
5. **Spike 375cir,** cSrdYNSASFSTFKsYGVSPTKcND*
6. **Spike 522cir,** CGPKKSTNLVKNKsVNFNFNcd*
7. **Spike 804-820cir,** cILPDPSKPSKRSFcgD*
8. **Spike 802-823cir,** FSQcLPDPSKPSKRSFcEDLLF*
9. **Orf1ab 1544-1564cir,** cFHLDGEVITFDNLKTLLSLREct
10. **Spike 462-501,** KPeERDgSTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTN

**Spike or S protein amino acid sequences represented by epitope peptides**

Numbered amino acid sequence for the S protein is shown below. (Sequences without breaks or spaces can be found in the Supplemental Materials section.)

Blue highlight shows the receptor binding domain (RBD), amino acids 319 to 529.[77] Red highlight shows amino acids that contact ACE2, the receptor for the virus. The transmembrane domain starts at residue 1209, near the C terminus of the protein sequence. Underlined portions of sequence show coverage by epitope peptides.

```
1    mfvflvllpl vssqcvnltt rtqlppaytn sftrgvyypd kvfrssvlhs
51   tqdlflpffs nvtwfhaihv sgtngtkrfd npvlpfndgv yfasteksni
101  irgwifgttl dsktqslliv nnatnvvikv cefqfcndpf lgvyyhknnk
151  swmesefrvy ssannctfey vsqpflmdle gkqgnfknlr efvfknidgy
201  fkiyskhtpi nlvrdlpqgf saleplvdlp iginitrfqt llalhrsylt
251  pgdsssgwta gaaayyvgyl qprtfllkyn engtitdavd caldplsetk
301  ctlksftvek giyqtsnfrv qptesivrfp nitnlcpfge vfnatrfasv
351  yawnrkrisn cvadysvlyn sasfstfkcy gvsptklndl cftnvyadsf
401  virgdevrqi apgqtgkiad ynyklpddft gcviawnshn ldskvggnyn
451  ylyrlfrksn lkpferdist eiyqagstpc ngvegfncyf plqsygfqpt
501  ngvgyqpyrv vvlsfellha patvcgpkks tnlvknkcvn fnfngltgtg
551  vltesnkkfl pfqqfgrdia dttdavrdpq tleilditpc sfggvsvitp
601  gtntsnqvav lyqdvnctev pvaihadqlt ptwrvystgs nvfqtragcl
651  igaehvnnsy ecdipigagi casyqtqtns prrarsvasq siiaytmslg
701  aensvaysnn siaiptnfti svtteilpvs mtktsvdctm yicgdstecs
751  nlllqygsfc tqlnraltgi aveqdkntqe vfaqvkqiyk tppikdfggf
801  nfsqilpdps kpskrsfied llfnkvtlad agfikqygdc lgdiaardli
```

---

```
851  caqkfngltv lpplltdemi aqytsallag titsgwtfga gaalqipfam
901  qmayrfngig vtqnvlyenq klianqfnsa igkiqdslss tasalgklqd
951  vvnqnaqaln tlvkqlssnf gaissvlndi lsrldkveae vqidrlitgr
1001 lqslqtyvtq qliraaeira sanlaatkms ecvlgqskrv dfcgkgyhlm
1051 sfpqsaphgv vflhvtyvpa qeknfttapa ichdgkahfp regvfvsngt
1101 hwfvtqrnfy epqiittdnt fvsgncdvvi givnntvydp lqpeldsfke
1151 eldkyfknht spdvdlgdis ginasvvniq keidrlneva knlneslidl
1201 qelgkyeqyi kwpwyiwlgf iagliaivmv timlccmtsc csclkgccsc
1251 gscckfdedd sepvlkgvkl hyt //
```

### Nucleocapsid or N protein

Only T-cell epitope peptides were selected from the sequence of the Nucleocapsid protein. Underlined portions of sequence show coverage by epitope peptides.

```
1    msdngpqnqr napritfggp sdstgsnqng ersgarskqr rpqglpnnta swftaltqhg
61   kedlkfprgq gvpintnssp ddqigyyrra trrirggdgk mkdlsprwyf yylgtgpeag
121  lpygankdgi iwvategaln tpkdhigtrn pannaaivlq lpqgttlpkg fyaegsrggs
181  qassrsssrs rnssrnstpg ssrgtsparm agnggdaala lllldrlnql eskmsgkgqq
241  qqgqtvtkks aaeaskkprq krtatkaynv tqafgrrgpe qtqgnfgdqe lirqgtdykh
301  wpqiaqfaps asaffgmsri gmevtpsgtw ltytgaikld dkdpnfkdqv illnkhiday
361  ktfpptepkk dkkkkadetq alpqrqkkqq tvtllpaadl ddfskqlqqs mssadstqa
//
```

## Combined B-cell and T-cell epitope peptides

**Spike 436-460** + **Spike 462-501; or Spike 436-460** + **Spike 462-476** + **Spike 478-502**
These combinations of peptides form a physical structure that reconstitutes much of the ACE2 interacting portion of the RBD. This stretch of amino acids contains mapped B-cell and T-cell epitopes identified in the immunodominance analysis of Zhang et al.[78] Other groups that mapped epitopes from convalescent sera did not identify these B-cell epitopes, but Zhang used longer peptides that are more likely to adopt conformations to allow antibody binding. Spike 436-460 provides a portion of the "450" epitope of Zhang, while Spike 462-501 (and Spike 462-476 + Spike 478-502) provides part of the 450 epitope and most of "480" from Zhang. These portions were mapped as B-cell epitopes, binding convalescent sera, and as T-cell epitopes by ELISpot assay. A 3D structure of the combined peptides is provided below.

---

[78] https://www.biorxiv.org/content/10.1101/2020.04.23.056853v2.article-info

**Spike 436-460** has multiple basic residues and has a positive charge of about +3 at neutral pH. No substitutions were made for this peptide. **Spike 462-501** and **Spike 462-476** each have multiple acidic residues.

**Spike 436-460, a.k.a. Spike1**

436 `NSNNLDSKVGGNYNYLYRLFRKSN`

IN TESTING, vaccines Generations 2, 3, 4, 5, 6, 7.

Within RBD, seven ACE2 contacting residues. Portion of Spike protein bound by antibodies in SARS-CoV and MERS-CoV.[79] In addition to the data of Zhang, this peptide spans two overlapping dominant T-cell epitopes predicted by Grifoni et al[80] and Braun et al[81]. This epitope also contains the experimentally verified homologous immunodominant T-cell epitope in SARS-CoV-1, Spike 436-443, YNYKYRYL (SARS-CoV-2 Spike 449-456)[82]. In this publication by Zhi and colleagues, peptides containing this epitope stimulated the strongest IFN-γ response in the ELISpot assay. Wang et al reported IgG antibodies to a stretch of this peptide. The sequence NYNYLYRLF is a top-scoring cytotoxic T lymphocyte (CTL) epitope predicted by the CTLpred server based on work by Basin and Raghava[83].

- Predicted T-cell epitope by Grifoni et al.: Class II, NLDSKVGGNYNYLYRLFR, 440-457
- Predicted T-cell epitope by Braun et al.: Class II, NLDSKVGGNYNYLYRLFR, 440-457

**Spike 462-501**

462 KPFERDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTN

**Spike 462-501** KP`f`ERD`g`STEIYQAGSTP`C`NGVEGFN`C`YFPLQSYGFQPTN
IN TESTING, vaccine Generation 6, 7.

This 40 aa peptide was ordered on May 9, 2020. Lower case highlighted letters indicate substituted amino acids. Native cysteines are highlighted in yellow, and are to be disulfide bonded (by charcoal treatment). The unmodified peptide was predicted to be marginally soluble, and these substitutions were made to increase solubility. The 40 aa Spike 462-501 peptide failed synthesis twice and was redesigned and made in modified parts as a 15 aa peptide (Spike 462-476) and a 25 aa peptide (Spike 478-502). These peptides arrived May 29. Spike 462-501 arrived June 5. We discussed the failure with the peptide synthesis team and they indicated that coupling efficiency in the amino acids `LQSYGF`, which are near the C terminus, contributed to the failed syntheses. They flagged Phe 497 as especially difficult. Therefore, the substitution

---

[79] https://www.biorxiv.org/content/10.1101/2020.02.22.951178v1.full.pdf
[80] https://pubmed.ncbi.nlm.nih.gov/32183941
[81] https://www.medrxiv.org/content/10.1101/2020.04.17.20061440v1
[82] https://www.sciencedirect.com/science/article/pii/S0042682205000620
[83] http://crdd.osdd.net/cgibin/ctlpred

Phe497His was made in Spike 478-502, which is predicted to be a moderately conservative substitution (Tyr was predicted to cause similar coupling inefficiency).

**Spike 462-476**: KPFERDISTEIYQAd
IN TESTING, vaccine Generations 5, 6, 7.

**Spike 478-502**: kPCNGVEGFNCYFPLQSYGhQPTNG
IN TESTING, vaccine Generations 5, 6, 7.

Spike 462-476 restores the substitutions made to Spike 462-501. The peptide pair Spike 462-476 and Spike 478-502 also contain a paired substitution of amino acids to increase solubility. The substituted amino acids come from the consensus amino acid sequence of SARS-CoV-1 Spike, which has a highly similar 3D structure. The C terminal Gly of Spike 462-476 was replaced with Asp and the N terminal Thr of Spike 478-502 was replaced by Lys. In the SARS-CoV-1 Spike 3D structure, these residues form a salt bridge. The intervening Ser 477 residue was omitted between the C terminus of  Spike 462-476 and the N terminus of Spike 478-502, to allow space for the carboxy and amino groups of these peptides.

These peptides contain multiple predicted B-cell and T-cell epitopes and span amino acid sequences of the C terminal portion of 450 and most of 480 from Zhang. Together with Spike 436-460, the total span is about 65 amino acid (a.a.) residues of the ACE2 binding portion of the RBD [84],[85],[86], and the highest scoring epitope from Fast and Chen. 3D modeling suggests Spike 462-501 (or the peptide pair Spike 462-476 and Spike 478-502) should form a complex with Spike 436-460, with the omission of Gly 461 to allow space for the amino group at the termini of this peptide, and carboxyl group at the C terminus of Spike 436-460. This combined structure contains a linear cluster of amino acids implicated in ACE2 binding of S protein by SARS and by SARS-CoV-2, as shown by Tian et al (Figure 1C)[87], Walls et al (Figure 2C), Park et al (Figure 2)[88], and Sun et al (Figure 1)[89]. The nucleotide sequences of multiple SARS-CoV-2 isolates show that the amino acids in this portion of S protein are highly invariant, indicating binding to the receptor depends on the amino acid composition of this epitope.

Solubility of Spike 462-501 is predicted to be marginal and was achieved by substitution: F464E and I468G. These are unlikely to play a role in antibody recognition. However, the sequence containing these substitutions has been identified as a T-cell epitope. Therefore, it would be ideal to restore these substitutions and achieve solubility another way, which was done for Spike 462-476.

---

[84] https://www.biorxiv.org/content/10.1101/2020.02.19.955484v1
[85] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7048180/
[86] https://www.ncbi.nlm.nih.gov/pubmed/?term=32155444
[87] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7048180/
[88] https://www.biorxiv.org/content/10.1101/2020.02.22.951178v1.full.pdf
[89] https://www.biorxiv.org/content/biorxiv/early/2020/02/20/2020.02.16.951723.full.pdf

- Predicted T-cell epitope by Grifoni et al.: 462-474; DRB1*04:01, KPFERDISTEIYQ
- Predicted T-cell epitope by Braun et al.:  462-474; MHC Class II, KPFERDISTEIYQ
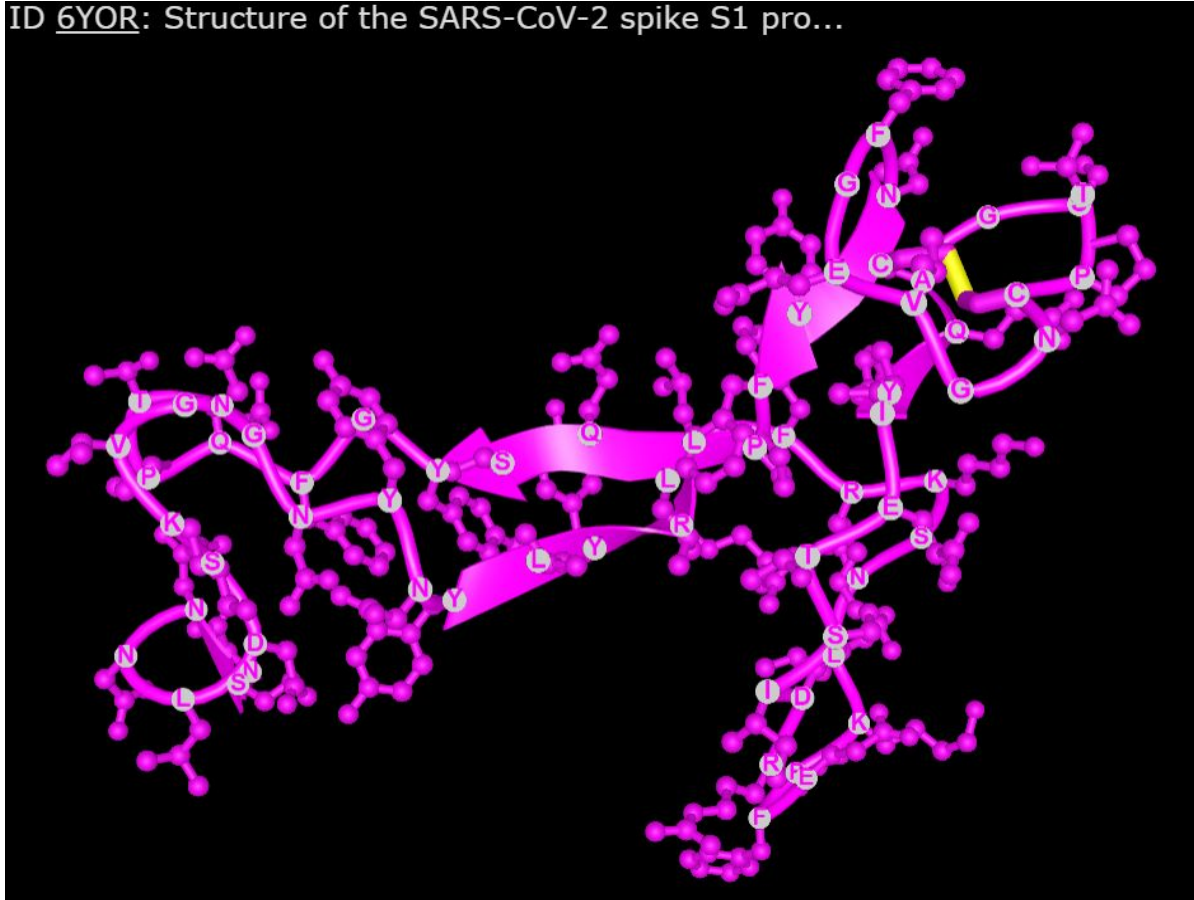- Peptide Spike 462-476: KPFERDISTEIYQAd



**Figure: Structure of the regions represented by Spike 436-460 + Spike 462-501 (and Spike 462-476 + Spike 478-502). Disulfide bond highlighted in yellow. The antiparallel beta strand shown is formed by the sections of each peptide rich in aromatic, polar, and hydrophobic residues, while most of the acidic and basic residues are clustered at right in a network of salt bridges. The top of this saddle-shaped structure contains the key residues for ACE2 binding.**

**Spike 550-574**

550  GVLTESNKKFLPFQQFGRDIADTTD

**Spike 550-574cir:** cgLTESNKKFLPFQQsGRDIADTcD
IN TESTING, vaccine Generations 3, 4, 5, 6, 7.
Lower case letters indicate substituted amino acids.

Provided the highest antibody signal in B-cell epitope mapping by Zhang et al. (see Figure 3). Spans one immunodominant epitope reported by Farrera et al[90]. Also, target of neutralizing convalescent antibodies from Poh et al., and overlapping B-cell predictions from Grifoni et al and Li et al. IgA of two convalescents of SARS-CoV bind the homolog of the N terminal half of this peptide. Disulfide circularized peptide. Cys1, Cys23 disulfide. Cysteines are inserted at positions 550 (G>C) and 573 (T>C), in order to create a disulfide bridge, preserving the looping structure and approximating the geometry of the central portion (`TESNKKFLPFQQ`) of the peptide shown in the figure below.  Substitution of Phe 565 with Gly (F565G). According to structural models, the side chain of Phe 565 projects into the interior of the protein and is therefore unlikely to play an essential role in antibody recognition. It lies in the center of a beta-strand that forms an antiparallel beta-sheet with amino acids 593 to 597. This part of the protein has been omitted and substitution of 551 (V>G) to prevent beta sheet formation between the two remaining beta strands that are separated in the native structure. This portion of the peptide is internal, away from the loop structure predicted to be bound by antibody. However, Braun et al used SARS homology to predict that this Phe is contained within a T-cell epitope.[91] Therefore, in future versions of peptides we recommend restoring this Phenylalanine: `cgLTESNKKFLPFQQFGRDIADTcD`.

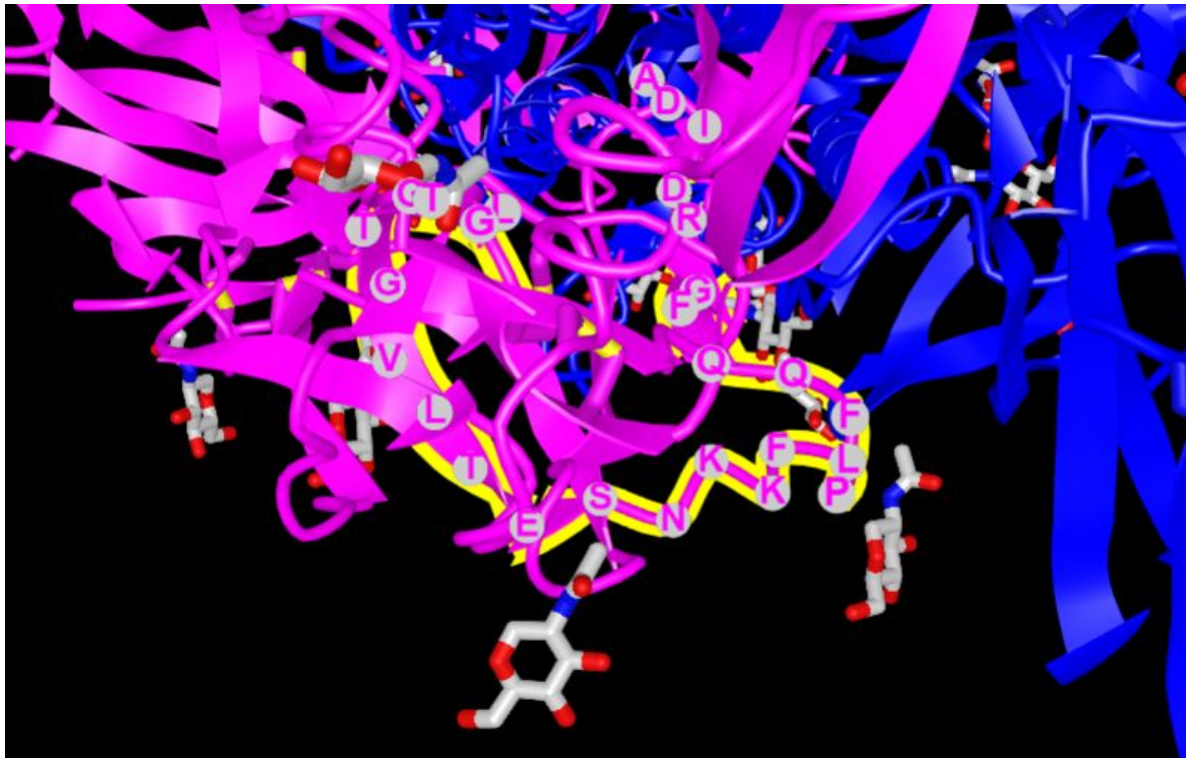- Predicted T-cell epitope by Braun et al.: MHC Class II,  SNKKFLPFQQ<mark>F</mark>GRDI, 555-569

[90] https://www.medrxiv.org/content/10.1101/2020.06.15.20131391v1
[91] https://www.medrxiv.org/content/10.1101/2020.04.17.20061440v1

**Figure: Surface loop structure of Spike 545-571 and Spike 551-573cir**

**Spike 375** One of the immunodominant epitopes identified by Zhang et al. Contains neutralizing residues bound by antibody CR3022. Multiple substitutions made outside the predicted T-cell epitope and antibody binding sites. Cysteine 389 in 3D image below substituted with serine (C389S). Tyrosine 375 and Leucine 397 substituted with Cysteines (Y375C, L397C) to form a disulfide bond for circularization. The native peptide sequence was predicted to be insoluble, so V377R and L378D are designed to provide solubility and preserve the conformation of the structure. Conservative amino acid substitutions are highlighted in green. This epitope also contains the experimentally verified homologous immunodominant T-cell epitope in SARS-CoV-1, Spike 366–374, CYGVSATKL (SARS-CoV-2 Spike 379-387)[92].

- Predicted T-cell epitope by Grifoni et al.: DRB1*07:01, DR8, SASFSTFKCYGVSPTKL, 371-387
- Predicted T-cell epitope by Braun et al.: MHC Class II, SASFSTFKCYGVSPTKL, 371-387

**Spike 375cir:** cSrdYNSASFSTFKsYGVSPTKcND
IN TESTING, vaccine Generations 4, 5, 6, 7.

---

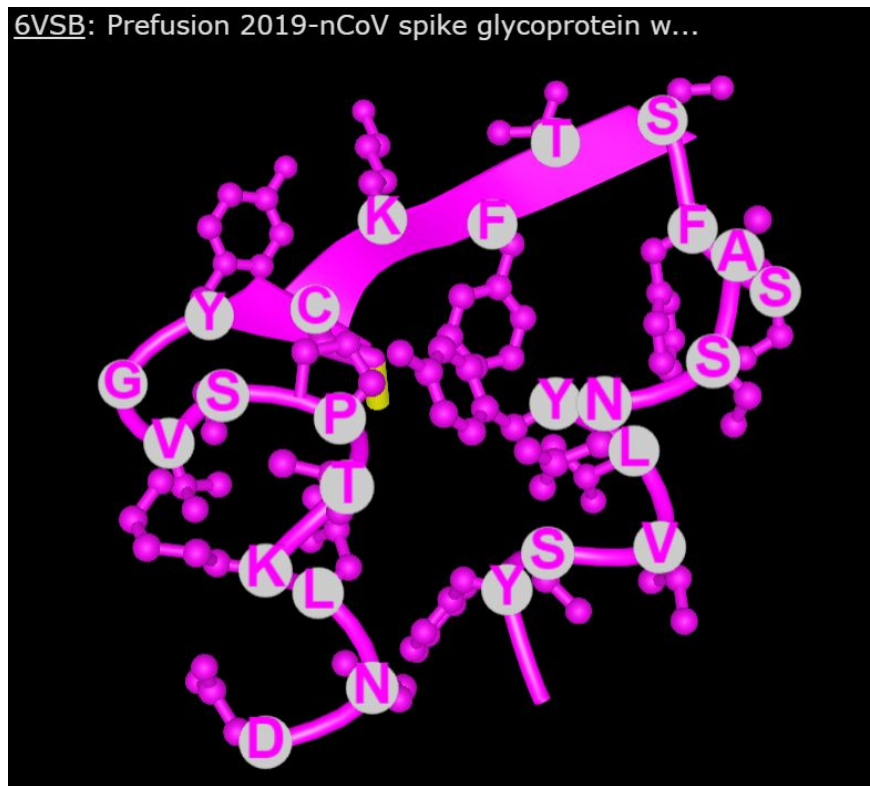[92] https://www.sciencedirect.com/science/article/pii/S0042682205000620

**Figure: Surface structure of Spike 375**

**Spike 522** ATVCGPKKSTNLVKNKCVNFNFNGL

One of the immunodominant epitopes identified by Zhang et al. This epitope also largely overlaps the experimentally verified homologous immunodominant T-cell epitope in SARS-CoV-1, Spike 525-532, VNFNFNGL (SARS-CoV-2 Spike 539-546)[93]. Multiple substitutions were made to circularize, to enhance solubility, and to provide a stabilizing salt bridge present in the full Spike protein. Cysteine 525 was retained and was made the N terminal residue. Glycine 545 was substituted by Cysteine to circularize (G545C) by disulfide bridge with C525. Cysteine 538 was substituted with Serine (C538S). Leucine 546 was substituted with Aspartic acid (L546D) to enhance solubility and to stabilize the structure. In the 3D structure, K528 forms a salt bridge with D389, and this C terminal D546 is predicted to substitute for D389.

- Predicted T-cell epitope by Braun et al.: MHC Class II, STNLVKNKCVNFN, 530-544

**Spike 522cir:** CGPKKSTNLVKNKsVNFNFNcd
IN TESTING, vaccine Generations 4, 5, 6, 7.

---

[93] https://www.sciencedirect.com/science/article/pii/S0042682205000620

## B-cell epitope peptides

### Spike protein epitopes

#### Spike 21

21  RTQLPPAYTNSFTRGVYYPDK
IN TESTING, vaccine Generation 7.

Immunodominant convalescent antibody-binding epitope described in linear epitope mapping studies by Wang et al, Zhang et al, and Li et al. An immunodominant neutralizing antibody maps to this region in SARS-CoV-1, and a neutralizing antibody binds this region in SARS-CoV-2[94]. This portion of the Spike protein is near the N terminus and is sufficiently unstructured as to not appear in currently available 3D structural models of the protein. This fact and the very strong signal in the linear epitope mapping study of Li et al suggest that this epitope peptide might reasonably approximate its native structure in the context of the full-length Spike, and thus is a good candidate for a peptide vaccine.

#### Spike 660

660  YECDIPIGAGICASYQTQTNSPRRA
IN TESTING, vaccine Generation 7.

This epitope lies immediately adjacent to the S1/S2 site that is cleaved by the host furin protease, which is required for subsequent cleavage at the S2' site by the host TMPRSS2 protease (Spike region 805-830 epitope immediately below). Immunodominant convalescent antibody-binding epitope described in linear epitope mapping  studies by Wang et al, Farrera et al, and Li et al. Neutralizing activity of antibodies that bind the S1/S2 site is hypothesized to be a result of reducing efficient cleavage of this site, preventing virus fusion with the host cell membrane[95].

#### Spike region 802-830

802  FSQILPDPSKPSKRSFIEDLLFNKVTLADA

Contains the site of the S2' cleavage site of the viral fusion peptide by the host TMPRSS2 protease, which is essential for membrane fusion and host cell infection. An immunodominant epitope bound by convalescent antibodies reported separately by Farrera et al, Poh et al, and Wang et al. Poh and colleagues demonstrated that this epitope elicits neutralizing antibodies[96]. Predicted B-cell epitope by Grifoni et al.. Contains the site of the S2' cleavage site of the viral fusion peptide by the host TMPRSS2 protease, which is essential for membrane fusion and host

---

[94] https://science.sciencemag.org/content/369/6504/650
[95] https://www.sciencedirect.com/science/article/pii/S1097276520302641
[96] https://www.nature.com/articles/s41467-020-16638-2

cell infection. Includes a top B-cell epitope prediction from Lon[97] and from Khan[98]. Multiple prolines, especially proline next to aspartate can cause problems with synthesis; however, as shown in the figure below, the loop accessible to antibody binding is largely determined by 3 proline residues. Furthermore, `ILPDPSKPS` is a homolog of the established T-cell epitope in SARS-CoV.[99]
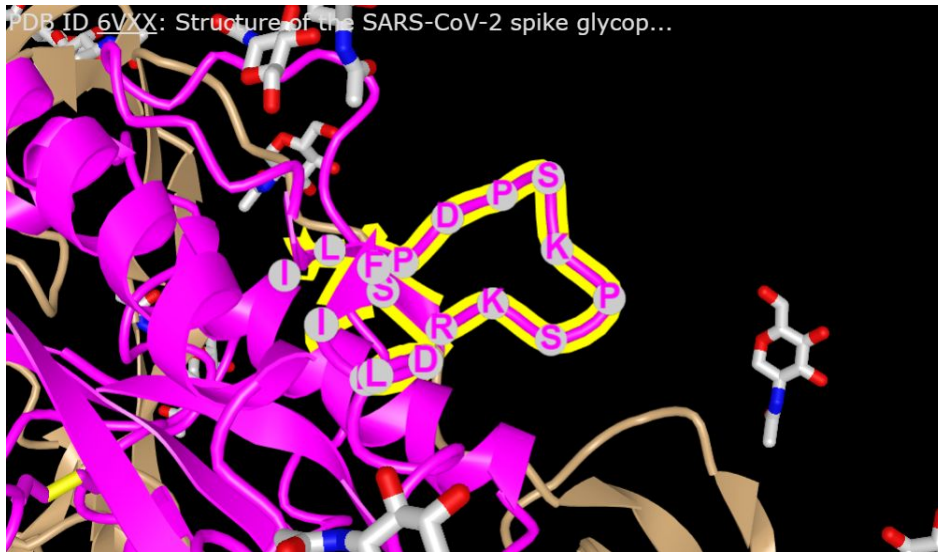


**Figure: Surface loop structure of Spike 805-830, Spike 802-823cir, and Spike 804-821cir, in the context of surrounding Spike residues and glycans.**

**Spike 804-820cir:** `cILPDPSKPSKRSFcgD`
IN TESTING, vaccine Generations 3, 4, 5, 6, 7. Cys1, Cys15 disulfide.
A Gly is added to the N term to facilitate contextual recognition of this epitope. Negatively charged Asp residue and C terminal carboxyl provide negative charges, and should help bind the peptide to the positively charged chitosan. Anchoring the peptide termini at both ends in this way might be a general strategy for synthesis of peptides, and additional acidic residues might be added to either or both N and C termini, as well as acetylation of the N terminus to neutralize its positive charge.

We also synthesized a second peptide variation for this epitope that restores the aspartic acid residue that forms a salt bridge with Lys 811 and Arg 815, and also contains more flanking residues that might be important for antibody binding:
**Spike 802-823cir:** `FSQcLPDPSKPSKRSFcEDLLF`
IN TESTING, vaccine Generations 5, 6, 7.

[97] https://www.biorxiv.org/content/10.1101/2020.04.03.022723v1
[98] https://www.biorxiv.org/content/10.1101/2020.05.03.074930v1
[99] https://www.sciencedirect.com/science/article/pii/S0166354216304016

**Spike 1145**

1145 `LDSFKEELDKYFKNHTSP`
<u>IN TESTING, vaccine Generation 7.</u>
Immunodominant convalescent antibody-binding epitope described in linear epitope mapping studies by Farrera et al and Li et al. This region was not covered in the studies by Zhang et al. This epitope is immediately adjacent to 50 aa heptad repeat 2 (HR2) fusogenic domain, which is essential for fusion of coronavirus and host cell membranes, and is 100% conserved between SARS-CoV-1 and and CoV-2 viruses[100].

## Nucleocapsid protein epitopes

Nucleocapsid is highly antigenic and produces large signals in ELISA of convalescent sera. Because of concerns about ADE, we are not using Nucleocapsid B-cell epitope peptides. If concerns about ADE risk are resolved, then these selections might be considered for use in a vaccine. However, as described below, certain portions of Nucleocapsid have been shown to be the longest-lasting T-cell epitopes, and we have selected these for eliciting lasting T-cell responses.

Nucleocapsid proteins of SARS-CoV-1 and CoV-2 display antibody binding immunodominance, and the portions bound are highly similar, which isn't surprising given the high degree of conservation between these proteins[101],[102]. The portions of Nucleocapsid that produce high signal in epitope mapping studies (for CoV-1) are amino acids 147-170 and 362-412. CoV-2 mapping by Wang et al produced very similar high binding signals for the C-terminal portion[103].
Mapping of CoV-1 N protein antibody binding epitopes shows no binding in the region 100-122, and low binding in 321-345, which represent the longer T-cell epitopes included in Gen 7+ vaccine. A CD8 T-cell epitope (at CoV-2 N 359-369) at the border of the C-terminal antibody binding region was selected for inclusion in Gen 8+ vaccine.

**T-cell epitope peptides**

**Nuc 100-120**

100 `KMKDLSPRWYFYYLGTGPEAG`
<u>IN TESTING, vaccine Generation 7.</u>

---

[100] https://www.thelancet.com/journals/lancet/article/PIIS0140673604157887/fulltext
[101] https://jcm.asm.org/content/42/11/5309.full
[102] https://www.biorxiv.org/content/10.1101/2020.03.26.994756v1
[103] https://www.biorxiv.org/content/10.1101/2020.03.26.994756v1

One of two extremely long-lived T-cell epitopes of patients recovered from 2003 SARS-CoV-1 infection, as described by Le Bert and colleagues[104]. T-cells from these patients are cross-reactive to homologous epitopes from SARS-CoV-2, which are highly conserved between the two viruses.

**Nuc 321-345**

321 `GMEVTPSGTWLTYTGAIKLDDKDPN`

IN TESTING, vaccine Generation 7.

One of two extremely long-lived T-cell epitopes of patients recovered from 2003 SARS-CoV-1 infection, as described by Le Bert and colleagues. T-cells from these patients are cross-reactive to homologous epitopes from SARS-CoV-2, which are highly conserved between the two viruses. Overlaps a predicted dominant T-cell epitope of Grifoni et al (`MEVTPSGTWL`, HLA restriction B$^*$40:01) [105].

**Orf1ab 1543-1576**

Candidate: 1543 `TFHLDGEVITFDNLKTLLSLREVRTIKVFTTVDN`

The two highest scoring T-cell epitopes predicted by Fast and Chen are clustered tightly together with a third on the Orf1ab protein. All three are spanned by a predicted epitope included in the vaccine design of Jain and colleagues[106] .

**Orf1ab 1544-1564cir:** `cFHLDGEVITFDNLKTLLSLREct`

IN TESTING, vaccine Generations 3, 4, 5, 6.

The highest scoring T-cell epitope predicted by Fast and Chen, and a second overlapping and high scoring T-cell epitope. MW 2768.2 g/mol. This peptide is predicted to be soluble; however, it is not water soluble at room temperature. Therefore, we did not disulfide circularize this peptide because it might further decrease solubility. And because much of the peptide is in the form of solid precipitate, separation from charcoal would not be simple and straightforward.

Other possible epitopes and peptides, including high solubility synthetic peptides are listed at the end of this document in the Supplemental Materials section.

## Preparation of chitosan nanoparticle vaccine

Published protocols exist for the robust production of chitosan nanoparticle vaccines. Various parameters for creating chitosan nanoparticles of specific sizes and characteristics have been studied and optimized. Ideal target nanoparticle size is in the range of about 100 nm to 200 nm, which is in the size range of many viruses, including SARS-CoV-2. Key parameters that influence particle size are chitosan concentration, chitosan to TPP ratio (charge ratio, or mass ratio,

---

[104] https://pubmed.ncbi.nlm.nih.gov/32668444/
[105] https://www.sciencedirect.com/science/article/pii/S1931312820301669
[106] https://www.biorxiv.org/content/10.1101/2020.03.26.009209v1

CS:TPP), salt concentration, and peptide or protein loading of the nanoparticle (higher load, larger size).[107]

Chitosan is a deacetylated form of chitin, which is found in mushrooms and the shells of crustaceans such as shrimp and crabs (seafood allergies are not allergies to chitin). Deacetylation produces free amino groups, and a high density of positive charges on chitosan. A solution of chitosan is mixed with negatively charged triphosphate (sodium triphosphate, STP a.k.a. tripolyphosphate, TPP) under rapid stirring or vortexing. Ionically bonded gel nanoparticles form spontaneously upon mixing with TPP, including when mixed together with peptide. The method here is adapted from published methods for creating peptide-loaded nanoparticles[108], and protein (ovalbumin) loaded nanoparticles.[109]

Chitosan (CS) of approximate average MW of 250 kDa (range 190 kDa - 310 kDa) was selected for preparation of nanoparticles. Deacetylation = 75% - 85%, average = 80%. Some publications report chitosan to TPP w/w ratios; however, degree of deacetylation can influence gelling behaviour and nanoparticle size. Therefore, parameters influencing nanoparticle characteristics have also been reported using charge ratios. Chitosan is a polymer composed of D-Glucosamine monomer subunits, and the molecular weight of each monomer is 161.16 Da. Therefore, for 80% deacetylated form, the average mass per positive charge is 161.16 Da/0.8 = 201.45. TPP has a MW of 367.86 Da and a charge of -5. Therefore, the average mass per negative charge is 73.57. An equimolar charge ratio (positive to negative charge) is achieved at a CS:TPP mass ratio of approximately 2.75:1.

Nanoparticles are smallest when the chitosan is most dilute. An ideal range of chitosan concentrations is 0.5 mg/ml to 1.5 mg/ml. Given the range of key factors tested in previous publications, a safe range for CS:TPP ratio (w/w) for 80% deacetylated chitosan is 7:1 to 9:1. Our initial trials focus on a ratio of 7.5:1. Hu et al report that a low CS:TPP ratio increases solution turbidity, probably by creating links between nanoparticles.[110] We have seen this phenomenon with a CS:TPP ratio of 4:1. A small amount of salt reduces particle size, probably by shielding adjacent amino charges in the chitosan. 50 mM NaCl keeps particle size compact and within range.

Chitosan to epitope peptide ratio (w/w). Ratios of chitosan:peptide have been reported in the literature from 1:1 to 25:1. The 1:1 ratio was achieved using chitosan/TPP as a carrier for insulin[111] and separately for ovalbumin. Loading efficiency decreases as the ratio approaches 1:1. Peptide loading of nanoparticles increases the size of the nanoparticles formed spontaneously. A 25:1

[107] https://scholar.google.com/scholar?cluster=18336291994796435345&hl=en&as_sdt=0,22
[108] https://www.frontiersin.org/articles/10.3389/fmicb.2015.00372/full
[109] https://www.ncbi.nlm.nih.gov/pubmed/?term=16380189
[110] https://scholar.google.com/scholar?cluster=10442461531761123020&hl=en&as_sdt=0,22
[111] https://pubmed.ncbi.nlm.nih.gov/10554100/

chitosan:peptide (w:w) results in approximately 50% increase in particle size, while a 10:1 ratio approximately doubles particle size. NaCl reduces particle size. 50 mM NaCl should allow for small particle size, and substantial loading of peptide into the nanoparticle. Initial target loading ratio of chitosan:peptide is 4:1.

Given that the vaccine is being used in pandemic conditions, to ensure that the vaccine is free of infectious virus contaminants during administration, solutions should be sterilized prior to mixing. Small volumes of chitosan/peptide and of TPP can be rendered free of infectious virus by heating in capped conical tubes to 55° C for at least 10 minutes, allowed to cool to room temperature, then mixed in a small beaker while magnetic stirring rapidly (>500rpm) for a few minutes. Per Amidi et al, 1% w/v Tween 80 can be added to assist solubility for high concentrations of chitosan and/or peptide.

**Stock solutions**

Once prepared, heat stock solutions to 55° C (15 minutes for 50 ml conical tube; 10 minutes for 15 ml conical tube) to ensure that they are free of infectious virus. 5 M NaCl does not require heat sterilization but can be treated at the same time for an added safety precaution.

- dH2O
- 5M NaCl (in dH2O)
- 3 mg/ml chitosan in 150 mM NaCl:
    - Add 48.5 ml dH2O to a 50ml conical tube
    - Add 1.5 ml 5M NaCl to the tube
    - Weigh 150 mg chitosan on a jeweler's scale, and add to the tube
    - Cap the tube and shake well until the chitosan is dissolved
    - Dilute to desired working concentration. To make 10 ml working concentration of 1.2 mg/ml chitosan in 60 mM NaCl, add 4 ml stock solution to 6 ml dH2O
- 1.0 to 1.5 mg/ml peptide solution(s). Mix peptides into dH2O
- 10 mg/ml $Na_5P_3O_{10}$ (sodium triphosphate, a.k.a. tripolyphosphate, TPP) in 100 mM NaCl
- OPTIONAL: solutions containing additional adjuvants

**Working solutions**

- 1.2 mg/ml chitosan in 60 mM NaCl
- 1.0 to 1.5 mg/ml peptide solution(s)
- 1 mg/ml TPP in 10 mM NaCl

**Mixing the vaccine - sample protocol to make about 25 doses of vaccine (about 12 doses plus priming waste)**

1. Heat the working solutions to 50 to 60 deg C (15 minutes for 50 ml conical tube; 10 minutes for 15 ml conical tube) to ensure they are virus free.
2. Sterilize the small beaker and stir bar by cleaning with soap and water, and then drying by wiping with an alcohol-soaked paper towel.
3. Place the beaker with the magnetic stir bar inside on a magnetic stir plate.
4. Add 7.2 ml chitosan working solution to the beaker.  Turn on the stir plate and slowly increase the stir speed to at least 500 rpm.
5. Add peptide solutions up to 1.5 ml (total of 1500 to 2500 micrograms peptide). If adding less peptide solution, make up the volume in dH2O to bring the total to 1.5 ml.
6. OPTIONAL adjuvant solutions should be added at this time.
7. Slowly add 1.3 ml TPP solution one drop at a time, and after the last drop is added continue to stir for at least 5 minutes.
8. Aliquot 800 microliters vaccine into sterile capped microfuge or 2 ml tubes.

## Administration of the vaccine

**Dosage amount.** Human studies have used as little as 7.5 micrograms inactivated virus or other replication incompetent antigen. Trials by El-Kamary et al. and Atmar et al. use 50 microgram and 100 microgram doses of viral-like particle in chitosan, although these trials use chitosan as a powder, which is a less efficient delivery vehicle than nanoparticles (see review by Smith et al. [112]). However, the intranasal inactivated influenza vaccine trial in healthy human adults conducted by Illum and colleagues used 7.5 micrograms and 15 micrograms per dose, with greater than double vaccine effectiveness (VE) at the higher dose[113]. We have used 50 micrograms to 100 micrograms total peptide for the initial priming and boost doses. Typical doses have been in the range of 70 micrograms. As of late July 2020, 20 self-experimenters have shown good tolerance for this dose. Assuming average coupling efficiencies for our synthetic epitope peptides of 70% to 80%, we expect the total peptide dose to be about 50 micrograms full length epitopes.

**Booster schedule.** The chitosan/peptide vaccine is only moderately immunostimulatory. This contributes to short-term safety, but requires higher and more doses for efficacy than injected vaccines with potent adjuvants. The vaccine should be used at least 3 times (as shown by animal studies and human clinical trial data): a priming dose, and administration of 2+ booster doses. People over the age of 60 or with complications that might attenuate immune response, might consider additional booster doses. Booster doses have been taken as soon as a few days after the prior dose, or as long as a few weeks. Ideally, doses are spaced by 1 to 2 weeks. Given the seriousness of the pandemic and individual circumstances, even shorter intervals between boosters might be used. This is done rarely even in a research setting, but most research is

---

[112] https://www.tandfonline.com/doi/full/10.4161/hv.27449
[113] https://www.sciencedirect.com/science/article/abs/pii/S0169409X01001715

conducted in support of eventual commercialization, and reflects those constraints. Considering the burden and level of stimulation that several weeks of CoV-2 infection exerts chronically on the immune system, more and frequent booster doses is not an unreasonable strategy. Over a dozen of RaDVaC have self-administered 4+ doses, respectively, of progressive generations of vaccine, spaced by at least a week each. Testing for immune response throughout the booster schedule and a few weeks after is ideal for monitoring building immunity; however, this is not practical for some people. Some have reported after-effects (e.g. nasal congestion) that increase with later booster doses. This is a positive qualitative sign of immune response, but even positive antibody and other test results should not be relied upon as a perfect proxy for immunity.

**Pre-administration.** To assess the pre-vaccination state of the immune system, one or more saliva samples, nasal wash or swab, and blood draws are ideal prior to vaccination.

**Administration.** Vaccination is achieved by nasal administration with the use of small (5ml to 20ml) commercial nasal sprayers. Depression of the sprayer top of our bottle delivers approximately 100ul of fine mist. This should be tested empirically by spraying into a small tube or beaker, and measuring by pipette. Dose is adjusted accordingly. Spray should be directed into each nostril. The sprayers we obtained come as a top sprayer unit, and either opaque white plastic or clear bottle. The sprayer unit stem stretches to the bottom of the bottle. The delivered volumes of vaccine will be in the range of 100 to 200 microliters per spray (total of 400 to 800 microliters), which is too small to be delivered reliably using the bottle. Therefore, we do not use the bottle for vaccination, and instead place the sprayer stem directly into the smaller vaccine vial. The stem of our sprayer unit is slightly longer than a 2 ml microcentrifuge tube. We pipette an aliquot of vaccine into the tube, then place the stem of the sprayer into the vaccine for spray administration.

**Protocol for vaccine administration**

To administer vaccine, it is ideal to wear gloves and have a spray bottle of 70% alcohol (ethanol or isopropanol) to sterilize your gloved hands, vials, and equipment. Select a well-lit work surface that can be sterilized with alcohol.

Required materials:

- Clean and well-lit work surface
- Gloves (nitrile, latex, etc)
- 70% alcohol (ethanol or isopropanol), preferably in a spray bottle
- Small (5ml to 20ml) commercial nasal sprayer bottle
- Vial of vaccine containing 1 dose plus extra for priming the sprayer (total, 800 microliter)
- Clean and preferably sterile paper towels or tissues
- OPTIONAL: saline wash solution and collection tube(s)

- OPTIONAL: rack or holder for vaccine vial

1. Sterilize the work surface with alcohol. Place a paper towel or tissue on the clean work surface, and spray with alcohol. You can place items on the surface and use the towel or tissue to dab or wipe items (e.g. the sprayer stem) while maintaining sterility.
2. Wipe the vaccine vial with an alcohol wipe. Loosen the cap so that it can be removed by lifting it off, but leave the cap in place, and set the vial upright on the work surface. If you choose to use a tube holder or rack for the vial, make sure it is sterilized.
3. Blow your nose thoroughly with a tissue or paper towel to clear your nasal passages. Use an alcohol soaked wipe or tissue to clean the outer area of the nose and just inside each nostril. This will help prevent inadvertent contamination of the sprayer tip with infectious virus that might be present on or just inside the nose. We do not recommend cleaning deep into the nasal passage; this is likely to do more harm than good.
4. Sterilize your spray bottle by placing 2 to 4 ml of 70% alcohol into the spray bottle, screw on the cap, and deploy the sprayer until a fine mist is sprayed.
5. Remove the spray top, and spray the residual alcohol. While you are doing this **DO NOT TOUCH THE SPRAYER TIP OR STEM** to maintain sterility. After sterilization or the following optional nasal wash, do not return the spray top to the bottle prior to using the vaccine.
6. **OPTIONAL**. You can use the sterilized spray top apparatus at this point to spray 100 mM NaCl into nostrils for collection of nasal wash samples. Spray saline into nostrils, inhale slightly, and then evacuate nasal wash into a collection tube. Collect at least 500 microliters.
7. While holding the spray top in one hand, uncap the vaccine vial with the other hand, and place the clean and sterile stem of the sprayer directly into the vaccine vial so that the stem touches the bottom of the vial.
8. Grasp the vaccine vial with one hand, and the spray top with the other hand so that your index finger and middle finger are on the side handles of the sprayer.
9. Deploy the sprayer into the air (not up your nose) until you see a fine mist, in order to clear residual alcohol and/or saline from the sprayer.
10. Insert the spray tip into a nostril. While inhaling through the nose, depress the sprayer (ideally, a total of about 100 to 150 microliters per spray). Sniff in the vaccine. Repeat the dosing, for a total of 200 to 400 microliters into each nostril. The entire procedure can take seconds or minutes but don't let the vaccine drip out of the nose, and don't blow your nose for at least an hour or two.
11. **OPTIONAL**. In later booster doses you can collect nasal washes or saliva after vaccination to assess immune response. At 4 hours post vaccination, collect the sample as described

in the previous OPTIONAL nasal wash. Cytokine levels in the sample can be measured as indicators of response, if you have the means to do this.

## Assessments of immunity

There are multiple important responses to an effective vaccine, and, therefore, multiple measures of effectiveness:

- B-cell/Antibody response
  - Neutralizing vs non-neutralizing
  - Neutralizing titer
  - Avidity/affinity of antibodies
- T-cell response
  - Cell mediated: Th1/Cytotoxic T Lymphocytes (CTL)
  - Humoral: Th2/CD4+

Neutralizing antibodies provide protection against viral infection and replication. They bind to key functional parts of the virus. Non-neutralizing antibodies can be beneficial by marking a virus for destruction, but they do not interfere with virus activities such as infection and replication, and they are also associated with ADE. High antibody titer is generally required for optimal protection. Avidity or affinity of the antibody binding to an antigen is another important measure of an effective antibody. An ideal B-cell response involves high-avidity neutralizing antibodies that bind to multiple viral epitopes. The sections below address details of measuring each of these key metrics.

### Samples to be collected and tested

- Whole blood and serum
- Nasal wash
- Saliva

Baseline samples will be collected, and then post-vaccination samples will be taken at booster timepoints, and also at later dates. Each of the above sample types has been used successfully to detect viral RNA and antibodies.

**Collection of samples for immune testing.** Ideally, all samples will be collected immediately prior to vaccination, and at various time points after initial and booster vaccinations.

**Blood** is ideally collected by venipuncture and vacutainer, but can be collected by lancet (finger) and deposited into sterile microcentrifuge tubes, without preservative or clotting chemistry. Antibody assays such as ELISA, typically start at dilutions of 1:100 or 1:200 and thus can be run on a few microliters of serum. About 50% of whole blood is recoverable as serum. Therefore, a few

drops of blood (30+ microliters) is sufficient for each individual at each time point, for each test. It is ideal to have enough serum for several tests. Blood and serum can be stored for relatively short periods (weeks) at —20°C until assayed. Whole blood or buffy coat are required for certain assays, such as ELISpot, that use peripheral blood mononuclear cells (PBMCs).

**Nasal wash**. Method adapted from de Fijter et al [114]. Use nasal sprayer to deposit 200+ microliters of sterile 100mM saline into one nostril.  Collect nasal wash effluent in a clean, labeled tube (a small funnel that fits into the collection tube is very helpful). Collection tube should be free of preservative, or contain non-denaturing preservative such as EDTA. Repeat the procedure with the other nostril. Repeat until you have collected a total of at least 0.5 ml. Don't collect more than 2 ml. Store at —20° C until further processed or assayed. Once all aliquots are collected, vortex with glass beads, then centrifuge the samples at 1000 g for 10 minutes at 4°C to clarify. Pipette the supernatants into fresh microcentrifuge tubes, and either refreeze at —20° C or process immediately.

**Saliva**. At least 0.5 ml should be deposited into sterile tubes, free of preservative, or containing a non-denaturing preservative such as EDTA.  Store at —20°C until assayed. Depending on dilution factor (typically starting at 10 fold or greater), assays can be run on a few microliters of saliva.


## Antibodies and B-cell immune response

Methods developed for assessing immunity to other respiratory viruses, such as influenza, can be adopted for use. Immunity will be assessed using standard indirect ELISA antibody assays (shown below) of serum, nasal swab or wash, and possibly saliva.

Variations of ELISA allow testing of the following:

- Presence/absence of antibodies that bind a viral specific antigenic target
- Antibody titer (by dilution series)
- Antibody avidity (e.g. by pulse of chaotrope, such as thiocyanate or urea)

While neutralizing antibodies can't be identified by ELISA, this information can be inferred from published information on which epitopes are bound by antibodies that show neutralizing activity. Thus, if a specific epitope peptide used in a vaccine is bound by an antibody, we can infer that it should be a neutralizing antibody without directly testing it in a neutralization assay.

Initial ELISAs will be performed using recombinant Spike RBD as the target antigen. A few of our B-cell epitope peptides lie within the RBD, and this region is highly represented in

---

[114] https://pubmed.ncbi.nlm.nih.gov/8872971

immunodominant neutralizing antibodies[115]. Followup ELISAs will be performed on various antibody fractions with individual epitope peptides in 96 well plates. Serum will be diluted 1/200, and plated in a standard 2X dilution series. Nasal washes are performed by use of a nasal sprayer and 100 mM saline, and the samples are analyzed by ELISA essentially as by Barria et al. [116] For a review of assessing the effectiveness of nasal vaccines for influenza, see Gianchecchi et al[117].
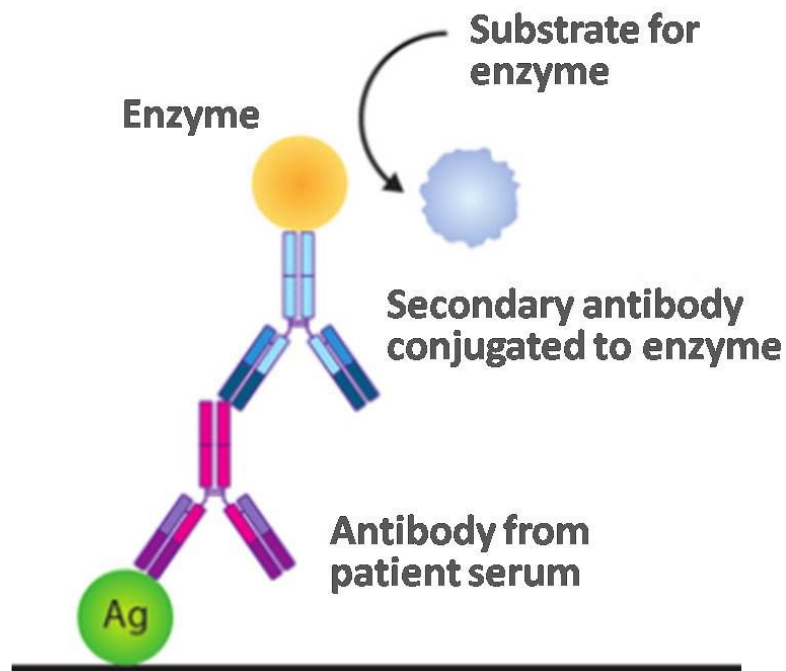


**Schematic of an indirect ELISA assay. Viral antigen (Ag) is attached to a surface (plate well). Patient serum is added to the plate well, washed and blocked. An enzyme-conjugated secondary antibody that binds to human antibodies is added to the plate well. A substrate for the enzyme is added to the plate well, producing a signal if the antigen is bound by antibodies in the patient's serum.**

A set of standard protocols and reagents for testing for SARS-CoV-2 virus exposure have been published.[118],[119] These protocols and reagents allow testing for immunity either elicited from our

---

[115] https://immunology.sciencemag.org/content/5/48/eabc8413
[116] https://academic.oup.com/jid/article/207/1/115/874878
[117] https://onlinelibrary.wiley.com/doi/full/10.1111/irv.12664
[118] https://labs.icahn.mssm.edu/krammerlab/wp-content/[...]expression-and-serology-protocol-3_24_20.pdf
[119] https://labs.icahn.mssm.edu/krammerlab/covid-19/

vaccine or from virus exposure. Slight modification of the protocols to use our peptides in ELISAs, and for testing of nasal washes, will allow us to test for immunity specific to the epitopes of our vaccine, and for nasal mucosal immunity.

Antibody avidity can be measured by multiple methods, as reviewed by Klasse[120]. In our vaccine schema, individual epitope peptides are used to elicit antibodies, and each of these can be used independently to assess avidity of binding antibodies.

## B-cell epitopes and ELISA antigen targets

### Spike protein epitopes / antigen targets

Purified recombinant Spike protein and Spike receptor binding domain (RBD) used as target antigen will allow for testing of viral exposure, and for immunity elicited by our vaccine, but will not be specific for immunity elicited by any specific epitope of our vaccine. Stadlbauer and colleagues use the recombinant RBD protein for testing rather than the full length Spike protein, due to low expression and purification of full-length Spike. We will use RBD as a primary target antigen.

Epitope peptides used in vaccine production can be used in ELISA and other tests for immune response. ELISA testing with recombinant RBD and separately with epitope peptides might help determine if immunity is due to the vaccine, or to virus exposure. ELISA with a protein other than Spike will be more definitive to distinguish between immunity from the vaccine, and immune response to virus exposure.

## Nucleocapsid protein B-cell epitopes / antigen targets

Nucleocapsid is widely used as a highly sensitive antigen target for antibody testing. We ordered a peptide of one of the most antigenic sequences of the highly antigenic Nucleocapsid protein (NP produces large signals in ELISA of convalescent sera). Because of concerns about ADE, we have not yet included Nucleocapsid B-cell epitopes in our vaccine designs. It is possible to use them as negative controls for ELISA. If concerns about ADE risk are resolved, then these selections might be considered for use in a vaccine. We have designed longer peptides, and shorter portions of this peptide.

**Nuc 371-399**

Longer candidate: `DKKKKADETQALPQRQKKQQTVTLPAAD`
Long single linear epitope near the C terminus of the Nucleocapsid protein. Substitution of Lys 379 to reduce the positive charge near the N terminus: Lys>Gly. This region has the longest linear epitope discovered by protein microarrays in SARS-CoV-2 (Wang) and SARS-CoV (Zhu) [121]. This

---

[120] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4766047/
[121] https://www.pnas.org/content/103/11/4011.long

epitope is split into two overlapping peptides, **Nuc 376-399** and **Nuc 357-382. Nuc 376-399** has an additional Glutamate and Alanine at the N terminus to reduce the positive charge and help associate with chitosan.

Substituted **Nuc 371-399:** `eaDKKKKADETQALPQRQKKQQTVTLPAAD`

**Nuc 357-382:** `IDAYKTFPPTEPKKDKKKKADETQAL`
Prospective peptide for synthesis
Overlaps with **Nuc 376-399**.

**Nuc 371-394cir:** Acetyl-`cDKKKKADETQALPQRQKKQQTecL`
SYNTHESIZED and ready for use as a negative control in testing
Nucleocapsid antibodies have been implicated in Antibody-directed enhancement. Take notice before selection for use in a vaccine. Useful as a negative control.
Cys1, Cys24 disulfide,
2958 g/mol. 2.96g/ml = 1M. 2.96mg/ml = 1mM 1mg/ml = 338uM

Use of the recombinant RBD as a standard for ELISA will not allow high confidence that an immune response is due to the vaccine rather than virus exposure. Use of envelope or other non-Spike SARS-CoV-2 proteins in ELISA will be more definitive. A positive result in either or both Spike-RBD and Spike1, but negative for non-Spike proteins is suggestive of immune response due to the vaccine.

Positive controls for infection should be obtained. Blood and others samples should be obtained from people who are or have been infected. They should have systemic IgG antibodies to not only Spike protein, but to other proteins as well. They might also have sIgA antibodies in nasal wash and saliva.

## T-cell immune response

There are many approaches to testing T-cell immunity specific to the virus. We are considering the following possibilities, and we welcome collaborations with scientists who can contribute to these and other analyses of T-cell responses.

- ELISpot[122]
- Transcriptomic profiling of PBMC[123,124]
- Immunome assessment

---

[122] https://en.wikipedia.org/wiki/ELISpot
[123] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3946932/
[124] https://www.ncbi.nlm.nih.gov/pubmed/?term=30205979

# SUPPLEMENTAL MATERIALS

### ADDITIONAL SEQUENCES

**Spike protein sequence without breaks or spaces, for peptide search:**

mfvflvlllplvssqcvnlttrtqlppaytnsftrgvyypdkvfrssvlhstqdlflpffsnvtwfhaih
vsgtngtkrfdnpvlpfndgvyfasteksniirgwifgttldsktqsllivnnatnvvikvcefqfcnd
pflgvyyhknnkswmesefrvyssannctfeyvsqpflmdlegkqgnfknlrefvfknidgyfkiyskh
tpinlvrdlpqgfsaleplvdlpiginitrfqtllalhrsyltpgdsssgwtagaaayyvgylqprtfl
lkynengtitdavdcaldplsetkctlksftvekgiyqtsnfrvqptesivrfpnitnlcpfgevfnat
rfasvyawnrkrisncvadysvlynsasfstfkcygvsptklndlcftnvyadsfvirgdevrqiapgq
tgkiadynyklpddftgcviawnsnnldskvggnynylyrlfrksnlkpferdisteiyqagstpcngv
egfncyfplqsygfqptngvgyqpyrvvvlsfellhapatvcgpkkstnlvknkcvnfnfngltgtgvl
tesnkkflpfqqfgrdiadttdavrdpqtleilditpcsfggvsvitpgtntsnqvavlyqdvnctevp
vaihadqltptwrvystgsnvfqtragcligaehvnnsyecdipigagicasyqtqtnsprrarsvasq
siiaytmslgaensvaysnnsiaiptnftisvtteilpvsmtktsvdctmyicgdstecsnlllqygsf
ctqlnraltgiaveqdkntqevfaqvkqiyktppikdfggfnfsqilpdpskpskrsfiedllfnkvtl
adagfikqygdclgdiaardlicaqkfngltvlpplltdemiaqytsallagtitsgwtfgagaalqip
famqmayrfngigvtqnvlyenqklianqfnsaigkiqdslsstasalgklqdvvnqnaqalntlvkql
ssnfgaissvlndilsrldkveaevqidrlitgrlqslqtyvtqqliraaeirasanlaatkmsecvlg
qskrvdfcgkgyhlmsfpqsaphgvvflhvtyvpaqeknfttapaichdgkahfpregvfvsngthwfv
tqrnfyepqiittdntfvsgncdvvigivnntvydplqpeldsfkeeldkyfknhtspdvdlgdisgin
asvvniqkeidrlnevaknlneslidlqelgkyeqyikwpwyiwlgfiagliaivmvtimlccmtsccs
clkgccscgscckfdeddsepvlkgvklhyt//

**Nucleocapsid protein sequence without breaks or spaces, for peptide search:**

msdngpqnqrnapritfggpsdstgsnqngersgarskqrrpqglpnntaswftaltqhgkedlkfprgqgvpintnss
pddqigyyrratrrirggdgkmkdlsprwyfyylgtgpeaglpygankdgiiwvategalntpkdhigtrnpannaaiv
lqlpqgttlpkgfyaegsrggsqassrssssrsrnssrnstpgssrgtsparmagnggdaalalllldrlnqleskmsgk
gqqqqgqtvtkksaaeaskkprq**krtatkaynvtqafgrrgpe**qtqgnfgdqelirqgtdykhwpqiaqfapsasaffg
msrigmevtpsgtwltytgaiklddkdpnfkdqvillnkhidayktf**pptepkkdkkkkadetqalpqrqkkqqtvtll
paadlddfskqlqqsmssadstqa**