# SARS-CoV-2 antibodies for specificity and function in clinical infection and asymptomatic cases: abridged secondary publication

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**KEY MESSAGES**

1. Antibody responses after SARS-CoV-2 infection are strongest against nucleocapsid, followed by ORF8, ORF3d, and ORF7a, surpassing the spike protein as a diagnostic target.
2. ORF8 is a sensitive and specific diagnostic target; use of combined antigens can further enhance specificity.
3. Children exhibit lower-magnitude antibody responses but demonstrate higher avidity and greater Fc receptor function per antibody. Their responses appear more specific and directly mature against SARS-CoV-2.
4. The Luciferase Immunoprecipitation System is a useful experimental approach for antigen discovery against novel outbreak viruses, particularly when reagents are limited and only viral RNA is available. This platform may be adapted for other viruses of interest.
5. Fc receptor functions of spike antibodies should be assessed after vaccination.

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## Introduction

SARS-CoV-2 virions comprise structural proteins. During infection, up to 30 additional proteins may be expressed based on putative open reading frames (ORFs) in the viral genome. Some of these proteins modulate cellular processes through direct interactions. Their truncations may influence disease pathogenesis and serve as antigenic targets for more specific serological assays. In addition to structural proteins, the ORF1a/b polyprotein and accessory proteins can elicit antibody responses during infection. Antibodies targeting non-structural proteins may affect viral infection via Fc-mediated effector functions and via interactions during virus entry, fusion, replication, and egress within infected cells. Because antibodies against non-surface proteins cannot directly mediate neutralisation, secondary Fc functions become critical. The balance of Fc binding is associated with COVID-19 outcomes. Characterisation of serological responses to these additional proteins provides a snapshot of the 'antibody landscape', including antibody magnitude, antigenic specificity, and biological relevance of SARS-CoV-2 proteins.

This study aimed to (1) develop a novel Luciferase Immunoprecipitation System (LIPS) assay as a diagnostic tool for clinical SARS-CoV-2 infection; (2) determine the immunodominance hierarchy of antibodies in asymptomatic SARS-CoV-2 infection; and (3) evaluate antibody effector functions targeting non-neutralising SARS-CoV-2 proteins.

## Methods

Based on previous studies describing the structure of the SARS-CoV-2 genome,[1,2] a panel of 14 proteins (S1, S2, S2', E, M, N, NSP1, ORF3a, 3b, 6, 7a, 7b, 8, and 10) was selected for antibody testing using the LIPS assay. Primers and cloning protocols for the amplification of SARS-CoV-2 proteins followed previously published methods.[3] Constructs containing the SARS-CoV-2 antigen of interest were cloned into the pREN2–Renilla luciferase (Ruc) plasmid, transfected into Cos1 cells, and prepared as previously described.[3]

The LIPS assays were performed with modifications[4] to established protocols.[3] Briefly, Ruc-antigens (at an equal concentration of $10^7$ per well) and heat-inactivated plasma (diluted 1:100) were incubated for 2 hours with shaking at 800 rpm. UltraLink protein A/G beads were added to the Ruc-antigen and plasma mixture and incubated for a further 2 hours with shaking at 800 rpm. The entire volume was then transferred to high-throughput screening plates and washed as previously described. Plates were then read and analysed. Experimental controls included blank wells without plasma but containing Ruc-antigens, as well as negative control

plasma from healthy donors collected prior to the COVID-19 pandemic. Background signal was defined as the luminescence detected from each Ruc-fusion antigen incubated with protein A/G and substrate in the absence of plasma.

Plates were coated with 80 ng/mL of spike, nucleocapsid, or ORF8 proteins. Plates were rinsed, blocked with 1% fetal bovine serum in phosphate-buffered saline, incubated with 1:100 heat-inactivated plasma diluted in 0.05% Tween-20 / 0.1% fetal bovine serum in phosphate-buffered saline for 2 hours, and rinsed again. To measure antibody FcγRIIIa binding, plates were instead coated with 500 ng/mL of protein, then incubated with 1:50 heat-inactivated plasma. Plates were subsequently incubated with biotinylated dimeric FcγRIIIa-V158 (2) at 50 ng/mL for 1 hour at 37°C, rinsed, and incubated with streptavidin-horseradish peroxidase (1:10 000). Horseradish peroxidase activity was detected using stabilised hydrogen peroxide and tetramethylbenzidine for 20 minutes, stopped with 2 M $H_2SO_4$, and read at 450 nm using an absorbance microplate reader.

## Results

From January 2020, at the onset of the pandemic, we cloned 14 different ORFs for application in the LIPS assay. Plasmids were shared internationally with collaborators in the United States, Germany, Singapore, and Australia, thereby facilitating global research efforts. Due to limited case numbers and sample volumes in Hong Kong prior to July 2020, we used 15 infected samples and pre-pandemic controls to establish the assay. The LIPS platform was successfully adapted for serological investigation of the SARS-CoV-2 immune response.

We conducted LIPS screening on hundreds of plasma samples from adults, children, asymptomatic individuals, and longitudinal collections, targeting 14 distinct SARS-CoV-2 protein regions. Pre-pandemic negative control samples were used to define assay sensitivity and specificity, with cut-off values set at the negative mean plus three standard deviations to ensure high stringency. Based on the mean difference compared with negative controls, the strongest antibody responses were observed against the nucleocapsid protein, followed by ORF8, ORF3b, and ORF7a. No difference in response magnitude was identified between symptomatic and asymptomatic cases. However, age-dependent variation in antibody response magnitude was evident, likely reflecting reduced viral replication in paediatric cases.

The most robust antibody responses were directed against the nucleocapsid and ORF8 proteins, which are abundantly expressed during infection but absent from the virion surface, in contrast to the spike protein. High-quality ORF8 protein was obtained. Protein-binding enzyme-linked immunosorbent assays were subsequently adapted to assess Fc receptor binding for spike-, nucleocapsid-, and ORF8-specific antibody responses, enabling evaluation of antibody effector functions. Fc receptor binding and antibody avidity against spike and nucleocapsid proteins were higher in children than in adults.

## Discussion

The relevance of antibodies targeting non-neutralising and internal proteins is increasing. Unbiased and quantitative platforms such as the LIPS assay offer valuable tools for serosurveillance in the post-COVID-19 era, allowing identification of key immunogenic targets in emerging viruses. Memory B cells specific to nucleocapsid and ORF8 proteins exhibit substantial maturation over time. However, monoclonal antibodies against these internal targets do not confer protection in vivo in murine models,[5] suggesting limited therapeutic potential despite having diagnostic value.

## Conclusions

SARS-CoV-2 infection induces an antibody response extending beyond the spike protein, which may improve diagnostic accuracy in both adults and children through the use of unique targets such as ORF8. FcγRIIIa effector functions are enhanced against spike in children relative to adults, and against nucleocapsid-specific responses, indicating a targeted immune response consistent with mild pathogenesis.

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## Disclosure

The results of this research have been previously published in:

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