A simple, sensitive, and low-cost FACS assay for detecting antibodies against the native SARS-CoV-2 spike protein

Julia Hambach¹ | Tobias Stähler¹ | Thomas Eden¹ | Dorte Wendt¹ | Natalie Tode¹ | Friedrich Haag¹ | Eva Tolosa¹ | Marcus Altfeld¹² | Anahita Fathi³⁴⁵ | Christine Dahlke³⁴⁵ | Marylyn M. Addo³⁴⁵ | Stephan Menzel¹ | Friedrich Koch-Nolte¹

¹Institute of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
²Department of Virus Immunology, Heinrich Pette Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany
³Section Infectious Diseases, I. Medical Clinic and Polyclinic, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
⁴Department of Clinical Immunology of Infectious Diseases, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany
⁵German Center for Infection Research, Partner Site Hamburg-Lübeck-Borstel-Riems, Hamburg, Germany

Correspondence
Friedrich Koch-Nolte and Stephan Menzel, Institute of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.
Email: nolte@uke.de (F.K.) and s.menzel@uke.de (S.M.)

Funding information
Deutsches Zentrum für Infektionsforschung, Grant/Award Number: 1.921; Deutsche Forschungsgemeinschaft.

Abstract
Background: Hamburg is a city state of approximately 1.9 Mio inhabitants in Northern Germany. Currently, the COVID-19 epidemic that had largely subsided during last summer is resurging in Hamburg and in other parts of the world, underlining the need for additional tools to monitor SARS-CoV-2 antibody responses.

Aim: We aimed to develop and validate a simple, low-cost assay for detecting antibodies against the native coronavirus 2 spike protein (CoV-2 S) that does not require recombinant protein or virus.

Method: We transiently co-transfected HEK cells or CHO cells with expression vectors encoding CoV-2 S and nuclear GFP. Spike protein-specific antibodies in human serum samples bound to transfected cells were detected with fluorochrome conjugated secondary antibodies by flow cytometry or immunofluorescence microscopy. We applied this assay to monitor antibody development in COVID-19 patients, household contacts, and hospital personnel during the ongoing epidemic in the city state of Hamburg.

Results: All recovered COVID-19 patients showed high levels of CoV-2 S-specific antibodies. With one exception, all household members that did not develop symptoms also did not develop detectable antibodies. Similarly, lab personnel that worked during the epidemic and followed social distancing guidelines remained antibody-negative.

Conclusion: We conclude that high-titer CoV-2 S-specific antibodies are found in most recovered COVID-19 patients and in symptomatic contacts, but only rarely in asymptomatic contacts. The assay may help health care providers to monitor...
Grant/Award Numbers: SFB1328-A10, SFB1328-A14, SFB1328-Z02

1 INTRODUCTION

Hamburg is a city state of approximately 1.9 Mio inhabitants in Northern Germany. The coronavirus disease 2019 (COVID-19) epidemic started in Hamburg in early March when families returned from their traditional skiing vacation and peaked in the middle of April with approximately 220 new cases per day. After having subsided in the summer months to less than five new cases per day, the currently resurging second wave of the epidemic has reached higher levels than during the first wave (≥500 new cases per day) (Figure 1). The accumulated total number of cases in Hamburg is approximately 16,000 (880 cases/100,000 inhabitants) with approximately 300 deaths (3% of cases) and 10,000 recovered patients (November 6th, 2020).2

Individuals in Hamburg that are tested severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) positive by polymerase chain reaction (PCR)-assay for coronavirus 2 (CoV-2) genomic RNA in throat swabs are referred to quarantine in their homes for 14 days together with other members of their household. Patients that develop severe disease are admitted to the University Medical Center Hamburg-Eppendorf or to another local hospital. Patients that develop respiratory failure are transferred to intensive care and those who go on to develop acute respiratory distress syndrome receive extracorporeal membrane oxygenation. Hamburg has had a surplus of intensive care unit-beds and respirators throughout the epidemic. Recovered patients and their household contacts are released from quarantine 2 days after recovery from symptoms. The lockdown in Germany is controlled in a step-wise fashion aiming to keep the number of new daily cases below 50/100,000 inhabitants. Recent analyses indicate that the degree of immunity in the general population is still below 2%.3

Several serological assays have been developed to detect CoV-2-specific antibodies.4–7 Most of these are based on enzyme-linked immunosorbent assays (ELISAs) that utilize recombinant proteins, e.g. the receptor-binding domain (RBD) of the spike protein (S). While the epidemic seems to be under control in China, it is resurging in most European countries and is still spreading rapidly in other regions of the world.8 Therefore, there is still an urgent need for serological tests with high specificity and lower costs.

Our lab has expertise in raising antibodies against native membrane proteins by complementary DNA (cDNA) immunization.9,10 We routinely use transiently transfected CHO or HEK293T cells to monitor specific antibody responses in immunized animals.11 Here we set out to apply this assay for detecting antibodies directed...
against the native spike protein of SARS-CoV-2 in human serum samples. The results demonstrate that this simple low-cost assay allows specific detection of CoV-2 S-specific antibodies. The assay can easily be set up in any research lab equipped with a cell culture facility and a fluorescent microscope or flow cytometer. The required plasmids and cells can be obtained freely from our lab.

2 | RESULTS

2.1 | Cotransfection of HEK or CHO cells with plasmids encoding the SARS-CoV-2 spike protein and nuclear GFP provides a simple tool to detect antibodies that recognize the spike protein in native conformation

To detect antibodies that recognize the spike protein in native conformation we transfected HEK or CHO cells with a full-length cDNA expression plasmid followed by immunofluorescence microscopy (IFM) or flow cytometry (Figure 2). Cotransfection with a cDNA expression plasmid encoding green fluorescent protein (GFP) fused to a nuclear localization signal permits distinction of cotransfected cells (green-fluorescent nuclei) and untransfected cells. The latter serve as negative controls that permit detection of antibodies directed against irrelevant cellular proteins. Cell-bound CoV-2 S-specific antibodies can be detected with a fluorochrome-conjugated secondary antibody. We use a 96-well format to simultaneously analyze many samples at a cost of less than 20 € per plate (see Table S1).

To validate the assay, we used the CoV-S-specific nanobody VHH-72\textsuperscript{12} in a human immunoglobulin 1 (IgG1) heavy chain antibody (hcAb) format\textsuperscript{13} (Figure 3). Bound hcAb was detected with PE-conjugated anti-human IgG (H + L). The results show that this antibody stains the vast majority of GFP-transfected cells but not GFP-negative cells (Figure 3A). The results indicate greater than 90% cotransfection of cells with the expression plasmids for GFP and CoV-2 S. The expression construct for nuclear GFP can be substituted with constructs for other suitable fluorescent proteins, for example, mitoDSRED or nuclear BFP, the secondary antibodies with other fluorescently labeled secondary antibodies, for example, fluorescein isothiocyanate or Alexa Fluor 647-conjugates (not shown).

2.2 | The vast majority of COVID-19 patients develop moderate to very high levels of CoV-2 S-specific antibodies

We next set out to monitor the antibody responses of SARS-CoV-2-infected individuals that had been hospitalized at the University Medical Center in Hamburg. We also analyzed serum samples from members of our lab that had continued to work during the epidemic while following the social distancing recommendations (lab work is organized in two shifts with coworkers wearing masks and maintaining a distance of >1.5 m). Sera were treated for 30 min at 56°C to inactivate complement components and SARS-CoV-2 virions. Figure 3B shows representative microscopy and flow cytometry results. The staining intensities allow a semiquantitative assessment of antibody levels by IFM (indicated by −, +, ++, +++).

![Figure 2](image-url) Schematic diagram of the assay. CHO cells are seeded onto a 96-well culture plate, HEK293 cells onto a tissue culture flask or petri dish. Cells are cotransfected with expression vectors for native full length CoV-2 S and nuclear GFP. Thirty-six hours after transfection, CHO cells are fixed for 10 min at RT on the plate with 2% PFA; 20 h after transfection HEK cells are harvested and transferred (non-fixed) to a 96-well plate. Cells are incubated for 30 min at RT with human serum samples. Cells are washed and bound antibodies are detected with PE-conjugated secondary antibodies. CHO cells are analyzed on the 96-well tissue culture plate by fluorescence microscopy with an inverse microscope equipped with a digital camera. HEK cells are analyzed by flow cytometry. CoV-2, coronavirus 2; PFA, paraformaldehyde; RT, room temperature
and a quantitative assessment by flow cytometry (indicated by the mean fluorescence intensity of GFP + cells). The results of IFM and fluorescence-activated cell sorting (FACS) analyses of representative 96-well plates are shown in Supplementary Figures 1 and 2, respectively. Cells were co-transfected with expression vectors for CoV-S and GFP at a 10:1 molar ratio, which could explain cells expressing CoV-S but little if any GFP.

Table 1 summarizes the results of the samples from hospitalized patients, Table S1 the results of samples from healthy coworkers. The results obtained by immunofluorescence microscopy and flow cytometry concord very well. Antibodies became detectable in all samples from hospitalized patients with 8–14 days after disease onset (Table 1). There is a slight tendency toward higher antibody levels in patients with more severe clinical symptoms. In contrast, all samples of healthy coworkers did not show any detectable antibodies (Table S1). We further analyzed 221 pre-COVID-19 samples with our FACS assay (Figure S3).

We used the reactivities of pre-COVID-19 samples to set a threshold (Figure 4). The results show that nine of 221
| Patient | Age | Days after disease onset | Disease severity | IFM | FACS | Plate |
|---------|-----|--------------------------|------------------|-----|------|-------|
| 1       | 73  | 14                       | ++++             | −   | 1.5  | 1D09  |
| 1       | 73  | 42                       | −                | +++ | 73.1 | 2F04  |
| 2       | 58  | 13                       | +++              | +   | 1.0  | 1E09  |
| 3       | 52  | 6                        | ++               | −   | 0.6  | 1E08  |
| 4       | 85  | 5                        | +++              | −   | 1.0  | 1D08  |
| 4       | 85  | 12                       | +++              | (+) | 8.0  | 1A08  |
| 4       | 85  | 41                       | −                | +++ | 56.2 | 2G04  |
| 5       | 60  | 9                        | +++              | −   | 0.9  | 1G09  |
| 6       | 57  | 10                       | ++               | (+) | 3.8  | 1C09  |
| 7       | 38  | 20                       | ++               | ++  | 23.1 | 1A12  |
| 8       | 50  | 8                        | ++               | +   | 0.9  | 1F09  |
| 9       | 63  | 11                       | +++              | ++  | 5.5  | 1B10  |
| 10      | 77  | 6                        | +++              | u   | 1.5  | 1H09  |
| 11      | 75  | 10                       | ++               | (+) | 6.2  | 1C10  |
| 12      | 29  | 7                        | ++               | ++  | 48.0 | 1A09  |
| 13      | 60  | 5                        | +++              | (+) | 2.0  | 1B09  |
| 13      | 60  | 12                       | +++              | ++  | 8.1  | 1F11  |
| 14      | 34  | 10                       | ++               | (+) | 4.6  | 2H09  |
| 14      | 34  | 17                       | −                | +   | 20.0 | 2F01  |
| 15      | 37  | 5                        | ++               | −   | 1.1  | 1A10  |
| 15      | 37  | 12                       | ++               | ++  | 4.9  | 1B12  |
| 16      | 61  | 4                        | ++               | −   | 0.6  | 1D10  |
| 17      | 73  | 9                        | +++              | (+) | 8.9  | 2H01  |
| 18      | 59  | 15                       | ++               | +++ | 54.5 | 2E02  |
| 19      | 57  | 10                       | +++              | ++  | 35.4 | 2E01  |
| 19      | 57  | 16                       | +++              | +++ | 65.2 | 2G03  |
| 20      | 38  | 4                        | ++               | −   | 1.0  | 1A11  |
| 21      | 27  | 10                       | ++               | +   | 9.2  | 2D01  |
| 21      | 27  | 28                       | −                | ++  | 43.3 | 2A04  |
| 22      | 42  | 8                        | ++               | +   | 2.9  | 2B02  |
| 22      | 42  | 16                       | ++               | +++ | 69.0 | 2H04  |
| 23      | 60  | 7                        | ++               | +   | 11.8 | 2C02  |
| 23      | 60  | 21                       | −                | +   | 11.7 | 2B04  |
| 23      | 60  | 31                       | −                | ++  | 9.1  | 2D06  |
| 24      | 47  | 4                        | +                | u   | 1.2  | 2G02  |
| 24      | 47  | 8                        | +                | u   | 2.7  | 2F03  |

(Continues)
tested pre-COVID-19 samples were false positive, that is, had values slightly above the threshold. Conversely, one of 33 samples from eight COVID-19 patients that were obtained more than 10 days after disease onset was false negative, that is, had a value slightly below the threshold. This corresponds to a calculated sensitivity of 97% and a specificity of 96%. Of the 14 samples from eight COVID-19 patients obtained less than 10 days after disease onset, three samples showed values above and eleven samples showed values below the threshold. The low levels of antibodies that bind the native spike protein of CoV-2 in some pre-COVID-19 samples may represent cross-reactive antibodies induced by a prior coronavirus infection.

2.3 | Eight of nineteen co-quarantined close contacts of COVID-19 patients did not develop any detectable CoV-2 S-specific antibodies

We next analyzed 60 samples from 27 individuals of eight households in which at least one member had contracted COVID-19 and had been co-quarantined with his/her family members or household contacts (Table 2, for asymptomatic household contacts, numbers in parentheses refer to the days after disease onset of their COVID-19+ household contacts. Further details on these households are provided in the supplementary materials). We also analyzed these 60 samples with two well-established ELISAs (Table 2). The results show very good concordance of our FACS assay with those of the Wantai and Euroimmune assays (Figure 5). Differences in FACS versus ELISA signal intensities observed for some samples likely are due to the different antigens employed in these assays. While our assay uses native, glycosylated spike protein displayed on the cell surface of living cells, the Wantai and Euroimmune employ subdomains, that is, the RBD domain and the S1 domain (the N-terminal and RBD domains) as purified recombinant proteins, respectively.

The eight members of households H1, H2, and H3 all reported symptoms and developed antibodies with one exception: H3-4 (19-year) did not recall any symptoms beyond a mild sore throat for 1 day and was repeatedly tested SARS-CoV-2 negative by PCR. He did develop CoV-2 S-specific antibodies, suggesting that he may have been the first member of his family to acquire

---

**TABLE 1 (Continued)**

| Patient | Age | Days after disease onset | Disease severity | IFM | FACS | Plate |
|---------|-----|-------------------------|-----------------|-----|------|-------|
| 24      | 47  | 21                      | −               | u   | 9.5  | 2C04  |
| 24      | 47  | 29                      | −               | u   | 8.3  | 2B05  |
| 25      | 65  | 3                       | +++            | −   | 0.9  | 2F02  |
| 26      | 78  | 0                       | +              | −   | 3.4  | 2H05  |
| 26      | 78  | 14                      | ++             | +++ | 67.1 | 2F07  |

*Note:* Data are from one experiment representative of two independent experiments.

Abbreviations: COVID-19, coronavirus disease 2019; FACS, fluorescence-activated cell sorting; IFM, immunofluorescence microscopy.

---

**FIGURE 4** Threshold, sensitivity and specificity of the FACS assay. (A) Antibody levels in 50 samples of COVID-19 infected individuals and 221 samples of pre-COVID-19 individuals were determined by flow cytometry using native spike protein expressed on the cell surface of CoV-2-S/GFP-cotransfected HEK cells. Samples obtained from COVID-19 patients within the first 10 days after disease onset are indicated by open circles, samples obtained from the same patients more than 10 days after disease onset are indicated by closed black circles. The threshold was set at the MFI of 200 (indicated by the long dashed line). (B) Specificity and sensitivity of the assay were calculated using samples obtained from COVID-19 patients more than 10 days after disease onset and all samples from individuals before December 2019. Data shown are from a single experiment and are representative of two independent experiments. COVID-19, coronavirus disease 2019; FACS, fluorescence-activated cell sorting; MFI, mean fluorescence intensity; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.
| Household | Age | Days after disease onset | Disease severity | IFM native S | FACS native S | ELISA S1 | ELISA RBD | Plate |
|-----------|-----|--------------------------|------------------|--------------|---------------|----------|-----------|-------|
| H1-1      | 64  | 7                        | ++               | –            | 0.5           | 0.34     | n.d.      | 2A07  |
| H1-1      | 64  | 10                       | +                | –            | 4.4           | 0.42     | 0.70      | 2H08  |
| H1-1      | 64  | 16                       | ++               | +            | 19.1          | 2.27     | 0.96      | 1F08  |
| H1-1      | 64  | 22                       | –                | ++           | 29.8          | 3.23     | 1.46      | 1C08  |
| H1-1      | 64  | 31                       | –                | ++           | 31.1          | 3.35     | 1.10      | 1H12  |
| H1-1      | 64  | 42                       | –                | +++          | 30.1          | 3.11     | 0.90      | 2E08  |
| H1-2      | 62  | 4                        | –                | –            | 0.8           | 0.40     | 0.04      | 2B07  |
| H1-2      | 62  | 16                       | –                | +            | 16.9          | 1.46     | 3.40      | 1B08  |
| H1-2      | 62  | 25                       | –                | +            | 18.0          | 2.37     | 2.67      | 1G12  |
| H1-2      | 62  | 31                       | –                | ++           | 23.2          | 3.28     | n.d.      | 2B08  |
| H1-2      | 62  | 36                       | –                | ++           | 24.1          | 0.93     | 3.35      | 2H03  |
| H2-1      | 41  | 61                       | –                | ++           | 8.3           | 2.46     | 3.10      | 2G07  |
| H2-2      | 35  | 57                       | –                | ++           | 11.0          | 2.09     | 1.80      | 2H07  |
| H3-1      | 50  | 24                       | –                | ++           | 23.3          | 3.75     | 3.84      | 1B05  |
| H3-2      | 56  | 14                       | –                | +            | 11.6          | 1.12     | 3.76      | 1A05  |
| H3-3      | 21  | 12                       | –                | +            | 10.6          | 2.22     | 3.78      | 1H06  |
| H3-4      | 19  | (24)                     | –                | ++           | 7.6           | 1.80     | 2.68      | 1G06  |
| H4-1      | 29  | 50                       | –                | +            | 11.7          | 2.53     | 2.26      | 2A05  |
| H4-2      | 26  | 45                       | –                | –            | 1.2           | 0.51     | 0.04      | 2H06  |
| H4-3      | 30  | 44                       | –                | +            | 11.3          | 2.08     | 1.03      | 2G06  |
| H4-4      | 25  | 41                       | –                | ++           | 18.5          | 2.98     | 3.06      | 2F06  |
| H4-5      | 26  | 32                       | –                | +            | 6.7           | 1.10     | 0.47      | 2E06  |
| H5-1      | 54  | 65                       | –                | ++           | 24.0          | 2.18     | 1.04      | 2H11  |
| H5-2      | 4   | (65)                     | –                | u            | 0.9           | 0.38     | 0.04      | 2G11  |
| H6-1      | 66  | 56                       | –                | +            | 18.5          | 3.10     | 3.78      | 2A11  |
| H6-2      | 60  | 56                       | –                | +            | 6.0           | 1.16     | 0.51      | 2H12  |
| H7-1      | 37  | 4                        | +                | –            | 0.8           | 0.43     | 0.04      | 1D11  |
| H7-1      | 37  | 7                        | +                | –            | 2.0           | 0.56     | 0.18      | 1E12  |
| H7-1      | 37  | 11                       | –                | u            | 4.8           | 0.48     | 0.30      | 2D09  |
| H7-1      | 37  | 17                       | –                | u            | 4.6           | 0.61     | 0.18      | 2C03  |
| H7-1      | 37  | 22                       | –                | –            | 4.0           | 0.76     | 0.28      | 2F05  |
| H7-1      | 37  | 37                       | –                | u            | 4.6           | 0.56     | 0.04      | 2C09  |
| H7-2      | 34  | –2                       | –                | –            | 0.7           | 0.32     | 0.03      | 1E11  |
| H7-2      | 34  | 1                        | +                | –            | 0.7           | 0.59     | 0.04      | 1F12  |
| H7-2      | 34  | 5                        | +                | +            | 3.2           | 0.35     | 0.22      | 2C01  |
| H7-2      | 34  | 11                       | +                | +            | 11.3          | 0.67     | 2.84      | 2D03  |
| H7-2      | 34  | 26                       | –                | ++           | 12.9          | 1.10     | 2.58      | 2G05  |

(Continues)
SARS-CoV-2, but had already eliminated the virus by the time of his PCR-tests. In households H4-H8, five members that reported symptoms also developed antibodies; one member (H4-2, 26-year) reported mild symptoms, was not tested by PCR and did not develop any detectable CoV-2 specific antibodies. The other eight members of H4-H8 did not show symptoms and did not develop any detectable antibodies.

### 2.4 Serial samples indicate maintenance of plateau antibody levels for up to 120 days

Serial samples were available from three SARS-CoV-2-infected hospitalized patients and from five SARS-CoV-2-infected household patients. We analyzed these samples in parallel in triplicates with our FACS assay using the mean fluorescence intensity as an indicator of the levels of antibodies against the native spike protein of SARS-CoV-2 (Figure 6). The results show a rapid rise in antibody levels within the first two weeks after disease onset. Antibody levels typically reach a plateau within 20–40 days. Most patients maintained a plateau antibody level for the duration of analysis (up to 120 days after disease onset). To assess the reproducibility of the assay, we performed independent repeat measurements of 10 samples covering the whole spectrum of reactivities from hospitalized patient H4 and household patient H 1-1 (Figure 6C). The results confirm a very good reproducibility with a calculated % coefficient of variation from 3.5 to 20.3 (mean 11.9).
Correlation of CoV-2 S-specific antibody levels in household patients assessed by FACS and ELISA. Antibody levels in 60 samples of 27 individuals (18 COVID-19-infected individuals and 9 co-quarantined contacts, Table 2) were determined by flow cytometry (native spike protein expressed on the cell surface of transfected HEK cells), Euroimmun ELISA (recombinant S1 domain), or the Wantai ELISA (recombinant RBD). Pearson correlation coefficients and p values (two-tailed) were determined using prism 8.4.3 (Graphpad). Interpolation was performed by robust line regression using prism 8.4.3. Dashed lines indicate the thresholds-borderline-spans for the respective assays. Data shown are from a single experiment and are representative of two independent experiments performed with the 60 samples of 27 individuals. COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting.

Antibody dynamics in eight patients. HEK293T cells were cotransfected with cDNA expression constructs for full length CoV-2 S and nuclear GFP as in Figure 3. CoV-2 S-specific antibody levels in (A) hospitalized patients and (B) household patients were assessed by flow cytometry in triplicate samples. Numbers refer to the patient assignments in (A) Tables 1 and 2, respectively. Error bars indicate standard deviation of three technical replicates. Data are from a single experiment that is representative of three independent experiments performed. (C) The ten samples of (A) patients 4 and (B) H1-1 were re-analyzed in five independent experiments. The results were used to calculate the average coefficient of variance between duplicates (% CV). cDNA, complementary DNA; CoV-2, coronavirus 2; CV, coefficient of variation; MFI, mean fluorescence intensity.
**3 | DISCUSSION**

We report a simple, low-cost assay for detecting antibodies against the native SARS-CoV-2 spike protein. The assay may help health care providers to monitor disease progression and antibody responses in vaccination trials, to identify health care personnel that likely are resistant to re-infection, and recovered individuals with high antibody titers that may be suitable as plasma and/or antibody donors.7,14–16

Low costs, simple set up, and incorporated controls to detect false positives, are the main advantages of our assay over established ELISA and peptide-based assays. Moreover, ELISA assays depend on the costly production and purification of recombinant proteins. Peptide arrays often miss antibodies directed against conformational epitopes. Use of purified plasmid DNAs and natively expressed spike protein likely account for the high specificity and reproducibility of our FACS assay. Co-transfection of cells with a cDNA encoding a fluorescent protein allows the clear distinction of “false positives” caused by antibodies that recognize irrelevant antigens. However, the FACS assay requires more time, equipment and operator skills than commercial ELISAs. It may thus more suitable for applications in research labs rather than for clinical diagnostic labs.

Our results with samples from hospitalized and household patients and their close contacts revealed that all individuals that had recovered from a symptomatic COVID-19 infection developed moderate to high titers of CoV-2 S-specific antibodies. Typically, antibodies became detectable 6–12 days after disease onset and reached a plateau level within three weeks. None of the analyzed laboratory personnel of the University Medical Center showed any detectable SARS-CoV-2 S-specific antibodies. Remarkably, eight of nineteen analyzed close contacts of COVID-19 patients in household quarantine did not develop any symptoms and also did not develop antibody mediated immunity even during co-quarantine with a symptomatic patient. This conclusion is in line with reports from other regional outbreaks of COVID-19.4,6,17,18

We conclude that individuals that have recovered from COVID-19 carry moderate to high titers of antibodies directed against the native CoV-2 spike protein. In contrast, many asymptomatic close contacts are antibody-negative. These individuals presumably continue to be susceptible to COVID-19 infection or might have a T-cell driven immunity from previous coronavirus infections.19

**4 | MATERIALS AND METHODS**

**4.1 | Serum samples**

Serum or plasma was obtained from patients and healthy volunteers after informed consent. For presentation in the tables, clinical symptoms were graded as follows: – none; (+) mild (fever, weakness); + moderate (headache, high fever, anosmia); ++ severe (requiring hospitalization); +++ life threatening (intensive care and respiratory aid). Further clinical information is provided as supplementary material to Table 2. Serum samples were incubated for 30 min at 56°C to inactivate complement and coronaviruses before use. All results shown in this paper were obtained with serum samples at a dilution of 1:100–1:200.

**4.2 | Expression constructs**

The codon-optimized intact open reading frame of the native spike protein of SARS-CoV-2 (GenBank MN908947) was produced by gene synthesis (pTwist-CoV2S). A similar construct (NR-52310) was kindly provided by the Krammer lab in New York. The expression construct for nuclear GFP (pCDNA6-GFPNls) was cloned in our lab as described previously by fusion of the coding sequences of GFP upstream of the DNA-binding domain of the transcription factor LKLF. Plasmids were purified with standard mini or maxi prep kits (Qiagen). These plasmids are available upon request from our lab.

**4.3 | Recombinant CoV-S-specific heavy chain antibody**

The codon-optimized open reading frame of the SARS RBD-specific nanobody VHH-72 flanked by NcoI and NotI restriction enzyme sites was generated by gene synthesis and cloned into the pCSE2.5 vector upstream of the hinge, CH2 and CH3 domains of human IgG1. VHH-72-human IgG1 hcAbs were generated by transient transfection of HEK-6E cells cultured for 6 days in serum-free medium. Cells were pelleted by centrifugation and hcAb-containing supernatants were stored at 4°C until use. Yield of recombinant hcAbs was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie-staining using 10 µl culture supernatant/well to be in the range of 20–200 µg/ml. Supernatants were used at a dilution of 1:20.
4.4 | Cells and cell transfections

Chinese hamster ovary cells (CHO) and human embryonal kidney cells (HEK293T) were obtained from the DSMZ, Braunschweig. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) medium supplemented with 10% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids and 1% glutamine. Aliquots of these cells are available from our lab. Cells were passaged every 2–3 days by trypsinization (Gibco) and passaged at a dilution of 1:4–1:10. A semi-confluent 10 cm petri dish or T75 culture flask is sufficient for transfection of a single 96 well plate. HEK cells were transfected in the T75 flask, CHO cells were passaged onto a 96 well plate before transfection. A stock solution of Polyethylimidine (Sigma) was prepared in sterile water (160 µg/ml). 10 µg of the SARS-CoV-2 S expression vector, 1 µg of the GFP expression vector, and 20 µg of PEI are sufficient for transfection of a 96-well plate. Plasmid DNAs were adjusted to a volume of 500 µl with 150 mM NaCl solution. Five hundred microliters of PEI solution (40 µg/ml in 150 mM NaCl) was added slowly to the DNA, vortexed for 10 s and rested for 20 min. For transfection of HEK cells, the entire DNA solution was pipetted dropwise onto the medium in the T75 flask using a 5 ml pipette; for transfection of CHO cells the DNA solution was distributed onto the 96 wells using a multichannel pipette (10 µl per well). Cells were incubated overnight and examined under an inverse fluorescent microscope for appearance of cells with a green fluorescent nucleus.

4.5 | Immunofluorescence microscopy

Twenty to 36 h after transfection, CHO cells were gently washed once with warm phosphate-buffered saline (PBS) and were incubated for 10 min in PBS containing 2% paraformaldehyde. The PBS/paraformaldehyde solution was discarded and replaced with 200 µl PBS containing 0.02% Na-Azide. Wells were used directly for IFM analyses or stored at 4°C for up to 6 weeks before use (wrap the plate with parafilm to prevent desiccation). Cells were incubated for 60 min at 4°C with serum diluted 1:10 in DMEM containing 10% fetal calf serum (FCS). Cells were washed twice with PBS and incubated further for 20 min at RT with PE-conjugated anti-human IgG (H + L) F(ab’)2 (Jackson) (diluted 1:200 in DMEM/FCS). Cells were washed twice with PBS and analyzed with an EVOS fluorescence microscope (Thermo Fisher Scientific) equipped with a digital camera.

4.6 | Flow cytometry

Twenty to 36 h after transfection, HEK cells were re-suspended by gentle pipetting in DMEM containing 10% FCS. Cells were washed by centrifugation, resuspended in PBS containing 0.2% BSA and distributed onto a round bottom 96-well plate (10^5 cells/well). Cells were incubated for 60 min at 4°C with serum diluted 1:200 in PBS/0.2% BSA, washed twice with PBS/0.2% BSA, and incubated further for 20 min at RT with PE-conjugated anti-human IgG (H + L) F(ab’)2 (Jackson) (diluted 1:200 in PBS/0.2% BSA). Cells were washed twice with PBS/0.2% BSA and analyzed with a FACS Celesta or a FACS Canto flow cytometer (BD Biosciences). Scores for IFM and FACS in Tables 1 and 2 were determined as described in Figure 3 (FACS MFI values were divided by 1000 for better legibility). Plate coordinates in Tables I and II correspond to those in Figures S2 and S3.

4.7 | ELISA

The EUROIMMUNE ELISA and Wantai ELISA were performed according to the manufacturers’ instructions. For the EUROIMMUNE ELISA, data is presented as a ratio of the optical density (OD) of the sample divided by the OD of an internal control (Calibrator) (ratio > 1.1 = “positive,” 0.8–1.1 = “borderline”, <0.8 = “negative”). The Wantai ELISA data is presented as a ratio of the sample OD divided by the “cut-off.” The cut-off is set to 0.19 if the average of the negative controls is below 0.03, which was the case in our assay.

4.8 | Statistics

Pearson correlation coefficients, p values (two-tailed) and coefficient of variation were determined using prism 8.4.3 (Graphpad). Interpolation was performed using line settings and robust regression using prism 8.4.3 (Graphpad). Triplicates were measured and plotted as mean ± SD using error bars on both sides.

4.9 | Study approval

The procedure followed in the study were approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg PV4780 and PV5139 for samples from healthy donors, and PV7298 for samples from COVID-19 patients). Written informed consent was obtained from recruited patients and healthy individuals.
ACKNOWLEDGMENTS
This study was supported in part by grants from the Deutsche Forschungsgemeinschaft to Eva Tolosa, Friedrich Haag, and Friedrich Koch-Nolte (SFB1328-A10, A14, Z02) and DZIF 1.921. We thank Josephine Gebhardt, Gudrun Dubberke, Marion Nissen, Kristina Colberg, Institute of Immunology, University Medical Center Hamburg-Eppendorf, Heike Hildebrandt, Department of Virus Immunology, Heinrich-Pette Institut, and My Linh Ly, Department of Clinical Immunology of Infectious Diseases, Bernhard-Nocht-Institute for Tropical Medicine, Hamburg, Germany, for excellent technical assistance. We thank H.-W. Mittrücker and B. Fleischer (Institute of Immunology, University Medical Center, Hamburg) for the critical reading of the manuscript. We thank Florian Krammer, Icahn School of Medicine at Mount Sinai, NY, USA, and Thomas Schirrmann, Technical University Braunschweig, Germany, for providing expression plasmids for CoV-2 S and for human IgG1 Fc, respectively. We thank the ID-UKE-COVID19 Study Group for samples and data analyses of COVID-19 patients. (ID-UKE-COVID19 Study Group: Marylyn M. Addo, Etienne Bartels, Thomas T. Brehm, Christine Dahlke, Anahita Fathi, Monika Friedrich, Svenja Hardtke, Till Koch, Ansgar W. Lohse, My L. Ly, Stefan Schmiedel, Yashin J. Simsek, Maya-Isabel Stolte, L. Marie Weskamm, Julian Schulze zur Wiesch; University Medical Center Hamburg-Eppendorf).

CONFLICT OF INTERESTS
All authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
Stephan Menzel and Friedrich Koch-Nolte contributed to completion of conceptualization and project administration. Tobias Stähler, Julia Hambach, Stephan Menzel, Thomas Eden, Dorte Wendt, and Natalie Tode contributed to investigation. Tobias Stähler, Julia Hambach, Stephan Menzel, Marcus Altfeld, Christine Dahlke, Anahita Fathi, Marylyn M. Addo, and Friedrich Koch-Nolte contributed to data analysis and data curation. Friedrich Haag, Eva Tolosa, Marcus Altfeld, Christine Dahlke, Anahita Fathi, and Marylyn M. Addo contributed to resources. Friedrich Koch-Nolte contributed to completion of writing. Friedrich Koch-Nolte, Stephan Menzel, and Marylyn M. Addo contributed to supervision. Friedrich Haag, Eva Tolosa, Marcus Altfeld, Marylyn M. Addo, and Friedrich Koch-Nolte contributed to funding acquisition. Open Access funding enabled and organized by Projekt DEAL.

DATA AVAILABILITY STATEMENT
Cells and plasmids are available from the Koch-Nolte-lab upon request (nolte@uke.de). The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Marcus Altfeld https://orcid.org/0000-0001-5972-2997
Friedrich Koch-Nolte http://orcid.org/0000-0003-1730-6674

REFERENCES
1. Pfefferle S, Kobbe R, Guenther T, et al. Infection control and virological assessment of the first cluster of COVID-19 in Northern Germany. SSRN. 2020.
2. Robert Koch-Institute. COVID-19 dashboard. https://www.rki.de/DE/Home/homepage_node.html. Accessed November 3, 2020.
3. Bannas P, Hambach J, Koch Nolte F Nanobodies and Camelid antibodies. 2020;181:1004-1015.
4. Danquah W, Meyer-Schwesinger C, Rissiek B, et al. Nanobodies that block gating of the P2X7 ion channel ameliorate inflammation. Sci Transl Med. 2016;8:366ra162.
5. Fumey W, Koenigsdorf J, Kunick V, et al. Nanobodies effectively modulate the enzymatic activity of CD38 and allow specific imaging of CD38(+) tumors in mouse models in vivo. Sci Rep. 2017;7:14289.
6. Eden T, Menzel S, Wesolowski J, et al. A cDNA immunization strategy to generate nanobodies against membrane proteins in native conformation. Front Immunol. 2017;8:1989.
7. Wrapp D, De Vlieger D, Corbett KS, et al. Structural basis for potent neutralization of betacoronaviruses by single-domain camelid antibodies. Cell. 2020;181:1004-1015.
8. Bannas P, Hambach J, Koch-Nolte F Nanobodies and nanobody-based human heavy chain antibodies as antitumor therapeutics. Front Immunol. 2017;8:1603.
15. Bloch EM, Shoham S, Casadevall A, et al. Deployment of convalescent plasma for the prevention and treatment of COVID-19. J Clin Invest. 2020;130:2757-2765.
16. Zhao Q, He Y Challenges of convalescent plasma therapy on COVID-19. J Clin Virol. 2020;127:104358.
17. Xiang F, Wang X, He X, et al. Antibody detection and dynamic characteristics in patients with COVID-19. Clin Infect Dis 2020.
18. Zhang B, Zhou X, Zhu C, et al. Immune phenotyping based on the neutrophil-to-lymphocyte ratio and IgG level predicts disease severity and outcome for patients with COVID-19. Front Mol Biosci. 2020;7:157.
19. Grifoni A, Weiskopf D, Ramirez SI, et al. Targets of T cell responses to SARS-CoV-2 coronavirus in humans with COVID-19 disease and unexposed individuals. Cell. 2020;181:1489-1501.
20. Batéjat C, Grassin Q, Manuguerra J-C, Leclercq I. Heat inactivation of the severe acute respiratory syndrome coronavirus 2. bioRxiv 2020.
21. Jäger V, Büsow K, Wagner A, et al. High level transient production of recombinant antibodies and antibody fusion proteins in HEK293 cells. BMC Biotechnol. 2013;13:52.
22. Schriewer L, Schütze K, Petry K, et al. Nanobody-based CD38-specific heavy chain antibodies induce killing of multiple myeloma and other hematological malignancies. Theranostics. 2020;10:2645-2658.
23. Zhang J, Liu X, Bell A, et al. Transient expression and purification of chimeric heavy chain antibodies. Protein Expr Purif. 2009;65:77-82.

SUPPORTING INFORMATION
Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Hambach J, Stähler T, Eden T, et al. A simple, sensitive, and low-cost FACS assay for detecting antibodies against the native SARS-CoV-2 spike protein. Immun Inflamm Dis. 2021;1-13. https://doi.org/10.1002/iid3.446