




RESEARCH ARTICLE

Over 90% of clinical swabs used for SARS-CoV-2 diagnostics contain sufficient nucleic acid concentrations

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Abstract

During the coronavirus disease 2019 pandemic caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), reliable diagnostics are absolutely indispensable. Molecular SARS-CoV-2 diagnostics based on nucleic acids (NA) derived from oro- or nasopharyngeal swabs constitute the current gold standard. Given the importance of test results, it is crucial to assess the quality of the underlying swab samples and NA extraction procedures. We determined NA concentrations in clinical samples used for SARS-CoV-2 testing applying an NA-specific dye. In comparison to cut-offs defined by SARS-CoV-2-positive samples, internal positive controls, and references from a federal laboratory, 90.85% (923 of 1016) of swabs contained NA concentrations enabling SARS-CoV-2 recognition. Swabs collected by local health authorities and the central emergency department either had significantly higher NA concentrations or were less likely to exhibit insufficient quality, arguing in favor of sampling centers with routined personnel. Interestingly, samples taken from females had significantly higher NA concentrations than those from males. Among eight longitudinal patient sample sets with intermitting negative quantitative reverse transcription polymerase chain reaction results, two showed reduced NA concentrations in negative specimens. The herein described fluorescence-based NA quantification approach is immediately applicable to evaluate swab qualities, optimize sampling strategies, identify patient-specific differences, and explain some peculiar test results including intermittent negative samples with low NA concentrations.

KEYWORDS

COVID-19, intermittent negative, nucleic acid quantification, RT-qPCR diagnostics, SARS-CoV-2, swab quality control

Abbreviations: ACE2, angiotensin-converting enzyme 2; BAL, bronchoalveolar lavage; CDC, centers for disease control and prevention; COVID-19, coronavirus disease 2019; C_t , cycle threshold; DEPC, diethylpyrocarbonate; E-, M-, N-, S-gene, envelope-, membrane-, nucleocapsid-, spike-gene; HCoVs, human coronaviruses; KCDC, Korea centers for disease control and prevention; LHA, local health authority; MERS-CoV, Middle East respiratory coronavirus; NA, nucleic acid; PCR, polymerase chain reaction; PHEIC, public health emergency of international concern; RT-qPCR, reverse transcriptase quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TE, tris-EDTA.

Robin L. Kligen and Benjamin Katschinski contributed equally to this work.

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1 | INTRODUCTION

As of today, over 107 million severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections have been confirmed and fatalities associated with the coronavirus disease 2019 (COVID-19) already exceeded 2.35 million (<https://coronavirus.jhu.edu/map.html>).¹ Based on regional undertesting and underreporting² as well as excess mortality calculations,^{3,4} the actual numbers of cases and fatalities must be considered to be much higher. Moreover, the global COVID-19 pandemic is far from being over.

Members of the *Coronaviridae* family cause widespread infections in mammals and birds. In addition to SARS-CoV-2, six further human-pathogenic coronaviruses (HCoVs) are known: four seasonal HCoVs (HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1) and the two emerging viruses SARS-CoV and Middle East respiratory coronavirus (MERS-CoV).⁵ The first SARS-CoV-2 outbreak was recognized in Wuhan, Hubei Province, China in December 2019.^{6–8} One month afterward, on January 31st, 2020, the World Health Organization declared the outbreak a public health emergency of international concern (PHEIC). Less than two months later, the PHEIC fulfilled the criteria of a global pandemic. Currently, 192 countries and regions are or have been affected by COVID-19 cases (<https://coronavirus.jhu.edu/map.html>). SARS-CoV-2 exhibits some clinical similarities to SARS-CoV but also some special features, such as the capacity to replicate in the upper respiratory tract enabling easier transmission.^{9,10} Hospitals and diagnostic departments responded to the pandemic by increasing the numbers of intensive care beds and ventilators as well as ramping up testing capacities. The United States Centers for Disease Control and Prevention (CDC) and similar institutions worldwide recommend reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for direct virus detection.^{11–13} Other diagnostic techniques include antibody detection and neutralization assays.¹⁴ Until now, 500 million SARS-CoV-2 tests have been conducted worldwide and around 4 million tests are currently performed per day.¹⁵ To mitigate the COVID-19 pandemic (colloquially referred to as “flattening the curve”), many countries had to or have to intervene with public life and economy by safety measures, such as reducing physical contacts to limit the number of newly infected people. Population- and risk-group-wide surveillance by swab sample collection and subsequent RT-qPCR diagnostics are the cornerstone of political decision making regarding the implementation as well as the lift of such restrictions. In several high incidence regions, negative test results are prerequisites for the permission to work, attend school, or enter the country. However, doubts concerning the effectiveness of mass screening efforts, especially in regions with low prevalence rates, have been raised.^{16,17} Additionally, cases have been reported in which patients received false-negative or intermittently negative SARS-CoV-2 test results.^{18,19} For example, a case report showed that despite the presence of typical COVID-19 symptoms, two swabs of the same patient tested negative for SARS-CoV-2 before the clinical manifestation was confirmed by bronchoalveolar lavage (BAL) sampling that tested positive.²⁰ Given the clinical, political,

and socioeconomic consequences and implications of such test results, it is of utmost importance to critically assess and continuously optimize sampling and diagnostic procedures.

In principle, one can use various types of clinical specimens for RT-qPCR diagnostics, such as swabs from naso- or oropharyngeal areas, sputum, stool, saliva, and BAL.²¹ Although recent reports discussed the potential of testing saliva samples,^{22,23} swabs are the most popular method used to acquire clinical specimens for routine diagnostics.²⁴ RT-qPCR primer pairs targeting different viral genes are in use. Typically, genes encoding the envelope (E), the spike (S), the membrane (M), the RNA-dependent RNA polymerase, or the nucleocapsid (N) protein are recognized by the primers and probes.²⁵ To minimize the rate of false-positive results, at least two, sometimes even three, viral genes are assessed to increase the test specificity.¹³ Commercial tests usually include polymerase chain reaction (PCR)-based amplification controls based on an exogenously added nucleic acid (NA) molecule and the corresponding primers to eliminate false-negative results based on PCR-inhibiting contaminations. One way to determine the quality of samples and RT-qPCR test results is to quantify a human housekeeping gene as internal references.^{26,27} However, since the availability of PCR test reagents is limited and the number of possible targets is restricted in most diagnostics devices, we aimed for an alternative approach to assess the quality of clinical specimens. To this end, we evaluated swab-derived eluates applying a fluorescence-based quantification of NA concentrations.

2 | MATERIALS AND METHODS

2.1 | RNA quantification and samples

Nucleic acids were extracted with the MagNaPure 96 large volume kit (Roche). NA was extracted from 500 μ l sample volume and eluted in 100 μ l elution buffer. A fraction of the eluate was used for direct virus detection using the RealStar SARS-CoV-2 RT-PCR kit (Altona), which targets the SARS-CoV-2 genes S and E. The remaining eluate was frozen at -20°C and thawed before NA quantification. NA concentrations were determined using the QuantiFluor RNA kit (Promega E3310, LOT: 20xTE 0000387719; RNA Dye 0000363041; RNA Standard 0000379699, 0000397566) in black see-through-bottom 96-well plates (Greiner Bio-One 655087, LOT: E20043TB). The eluted NA was measured according to the manufacturer's instructions. In brief, sequential twofold dilutions of the provided RNA standard (7.8–500 ng/ml) were prepared in tris-EDTA (TE) buffer. The QuantiFluor RNA dye was diluted in a ratio of 1:400 (vol/vol) in TE buffer and 200 μ l of the dilutions were pipetted into the wells of a 96-well plate. The TE buffer was provided in the QuantiFluor kit as a 20-fold stock solution and was diluted with DEPC-treated H_2O (Carl Roth T143.2, LOT: 419289584). Subsequently, 10 μ l of the eluted NA was added per well and incubated for 5 min in the dark at room temperature. Fluorescence (492 nmEx/540 nmEm) was measured using a Mithras 943 Multimode Plate Reader (Berthold). All measurements were performed in duplicates. A blank solution was used

to determine the background fluorescence that was subsequently subtracted from the mean of the duplicate values. Using this protocol, the testing of 40 individual samples on one 96 well plate (40 samples in duplicates + controls) takes less than one hour. The reagent and disposables cost approximately less than 0.25 € per sample.

Sampling procedures conducted by public health authorities of the city of Essen were performed by personnel trained by the Bio Task Force and Jörg Spors (Essen Fire Department) supported by Bastian Brune (Essen Fire Department and University Hospital Essen).

Assessment of test samples for the improvement of diagnostic procedures has been approved by the ethics committee of the medical faculty of the University of Duisburg-Essen (20-9512-BO).

2.2 | Round-robin test samples

The round-robin test samples by INSTAND e.V. are internationally used for quality control of SARS-CoV-2 testing laboratories. The samples consist of lyophilized cell culture lysates, containing a defined number of viral genomes.²⁸ Since the samples supposedly contain less cellular NA, they served to determine the lowest NA concentration cut-off value.

2.3 | Internal positive control

The internal positive control used by the diagnostics department of the Institute for Virology of the University Hospital Essen consists of pooled samples derived from 38 positive tested patients. After pooling, the samples were tenfold diluted with media. This control is used in every extraction and RT-qPCR for SARS-CoV-2. Out of 38 samples, one sample was a BAL and the remaining 37 were swabs. The average age of the patients the samples were taken from is 52 years (ranging from 23 to 94 years). They were 20 (52.6%) females and 18 (47.4%) males. In total, we measured 72 independent NA extractions of the positive control. The average NA concentration was 2.43 ng/μl, ranging from 1.32 to 9.01 ng/μl.

3 | RESULTS

Based on the continuous debate concerning the reliability and predictive value of SARS-CoV-2 tests and whether and how the quality of collected specimens might influence test performances, we aimed to implement a fast, cost-effective, and convenient NA quantification protocol. Given the limitations of various diagnostic RT-qPCR cyclers in terms of the capacity to multiplex more fluorescent probe sets, and the demand for parallel tests recognizing several respiratory viruses in addition to SARS-CoV-2, such as influenza viruses, we opted against an additional RT-qPCR primer set identifying a housekeeping gene. Instead, we set out to quantify the overall NA

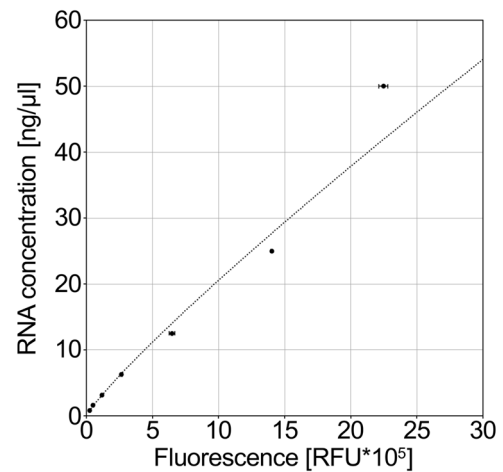


FIGURE 1 Establishment of QuantiFluor RNA assay. The QuantiFluor assay high concentration standard was used to calculate a power regression curve. A serial dilution of the manufacturer's RNA standard was measured at the concentrations of 50, 25, 12.5, 6.25, ~3.12, ~1.56, and ~0.78 ng RNA/μl. Fluorescence in relative fluorescence units (RFU) was quantified with a plate reader. $r = .992$; $r^2 = 0.984$, $p < 1.69E^{-23}$. Average of duplicates \pm SD is shown

concentration using the commercially available RNA-specific dye QuantiFluor combined with a set of well-defined RNA concentration standards as calibrators (see Section 2 for details). As expected, the measured fluorescence intensities correlated very well with known RNA concentrations (Figure 1; $r = .992$; $r^2 = 0.984$, $p < 1.69E^{-23}$). Based on the pre-screening results comprising a few prototypical clinical specimens, we chose to apply the manufacturer's protocol for high RNA concentrations (data not shown).

After the successful establishment of the NA quantification method, we determined nucleic acid concentrations in a comprehensive set of clinical specimens submitted to our routine diagnostics department for RT-qPCR-based SARS-CoV-2 testing. All samples had been collected between March and June 2020 at various clinics and departments of the University Medicine Essen and at nearby hospitals in the state of North Rhine-Westphalia, by public health authorities of the city of Essen, by the staff of the central emergency department of University Hospital Essen or by other personnel. We analyzed the NA concentrations in 1234 eluates after automated NA extraction (see Section 2 for details). The vast majority (1138; 92.22%) of NA eluates was derived from naso- or oropharyngeal swabs. The remaining 96 samples (7.78%) were either BAL (51 samples), sputum (8 samples), or stool (16 samples). Additionally, we measured samples without an accurate sampling description (20 samples) and the eluate of a sample taken from a bronchus during organ transplantation (1 sample). The average NA concentration of swabs was 17.76 ng/μl with a range of 0.1–58.09 ng/μl. BALs exhibited an average NA concentration of 27.03 ng/μl (range: 1.78–58.40 ng/μl), sputum samples an average of 24.54 ng/μl (range: 14.58–38.18 ng/μl), stool samples an average of 4.26 ng/μl (range: 0.43–18.82 ng/μl), specimens collected at undocumented localization had an average NA concentration of 18.87 ng/μl (range:

1.33–38.25 ng/μl), and the bronchus-derived eluate had a concentration of 20.59 ng/μl (data not shown). Since they make up the majority of samples and are the most widespread material used for SARS-CoV-2 testing, we focused solely on swabs in our further assessments.

Swabs were obtained from individuals with a broad age range of 1 day–99 years (median age: 63 years). Approximately 44% (495) of samples were derived from females. Among all swabs, 1016 (89.28%) tested negative for the SARS-CoV-2 S and E gene, whereas 122 (10.72%) had positive RT-qPCR results (116 were double positive; 2 were single positive for the E gene; 4 were single positive for the S gene). Since we purposefully included SARS-CoV-2-positive samples, this percentage does not provide information concerning the overall prevalence of SARS-CoV-2 positivity in the local population. After we had established a simple method to quantify NA concentrations, we intended to evaluate the sample quality in comparison to relevant concentration benchmarks. To this end, we applied three different cut-off values based on the minimal NA concentration among (I) 122 positive samples sufficient to provide an unequivocally positive RT-qPCR result, (II) in-house positive controls included in each RT-qPCR run, and (III) well-defined samples of a federal round-robin laboratory comparison. In the group of swabs with a positive RT-qPCR result, 2.93 ng/μl was the minimal NA concentration. Only 9.15% (93) of negative samples had lower concentrations. The swab with a NA concentration of 2.93 ng/μl was obtained from a 65-year-old woman. RT-qPCR testing of this sample reached a cycle threshold (C_t) value of 34 for the S and the E gene. For the 93 specimens with NA concentrations below the cut-off, we cannot reliably distinguish between true absence of SARS-CoV-2 and false-negative results due to improper sampling, transport or NA extraction. All samples were collected in June 2020. At this time, less than 2% of surveillance tests performed in the local German population were positive for SARS-CoV-2.²⁹ Although there may be a bias towards higher incidences when specimens are taken at hospitals or by local health authorities, statistically, only very few these 93 indefinable specimens should have been truly positive for SARS-CoV-2 by chance. Nevertheless, we assessed if the corresponding patients had been tested during a period starting 14 days before the doubtful sampling and ending 14 days afterwards. Fortunately, none of the patients from whom the specimens with an NA concentration below 2.93 ng/μl were collected, tested positive for SARS-CoV-2 at the University Hospital Essen during this period. In 51.61% (48 samples), a subsequent SARS-CoV-2 test for SARS-CoV-2 was negative within 14 days of the initial sampling. In 73.12% (68 samples), a negative test result preceded or succeeded the sample with an NA concentrations below the 2.93 ng/μl cut-off during the 28 day period, suggesting that repeated sampling may reduce the risk to miss SARS-CoV-2 infections.

Our next lower cut-off value in the group of the in-house positive controls was 1.32 ng/μl. Only 3.64% (37) of samples which tested negative for SARS-CoV-2 had lower NA concentrations. Cell culture-derived virus samples of the federal round-robin laboratory test had a minimal NA concentration of 0.96 ng/μl. Only 3.05% (31)

of samples tested negative had a NA concentration below this lowest cut-off (Figure 2A). Even if the least stringent lower cut-off value is applied as a reference, 90.85% of negative samples exhibited NA concentrations that would have allowed an RT-qPCR-based SARS-CoV-2 recognition as judged by positive results in parallel tests. The NA concentrations of the SARS-CoV-2-positive swabs were in a narrow range (~3–39 ng/μl; ~13-fold difference). Assuming an optimal 2-fold amplification per PCR cycle, a 13-fold difference corresponds to approximately 3.7 C_t values. Given that the actual C_t values ranged from 12 to 44, NA concentrations in the specimen can only influence the quantitative result within narrow boundaries. Accordingly, we neither found a significant correlation of the NA concentrations with the C_t values determined by RT-qPCRs for the S ($r^2 = 0.024$; $p = .06$) nor for the E gene ($r^2 = 0.019$; $p = .092$) (Figure S1), suggesting that an NA quantification method can identify samples with insufficient NA concentrations but is not able to predict viral loads, which are influenced by numerous factors, such as the patient's individual susceptibility, the therapy, and the course of disease.

We stratified the samples by criteria, such as age, sex, and sample origin to evaluate donor-specific differences. Age groups were assigned according to the grouping used by Germany's federal disease control and prevention agency, the Robert Koch Institute, for example on its COVID-19 dashboard.³⁰ We did not find a correlation between patient age and NA concentrations in swab-derived samples (Pearson's $r = .097$; data not shown). The rate of samples with less NA than 2.93 ng/μl ranged from 0% to 13.13% in different age groups (Figure 2B).

Although no statistical significance was reached in the individual age cohorts, all groups showed a trend towards higher average NA concentrations in swab samples obtained from female patients as compared to male patients in the same age group (Figure 2B). However, when we compared all samples derived from males and females against each other to evaluate potential sex-related differences concerning NA concentrations in swab samples, we observed a significantly ($p = .03$) higher median nucleic acid concentration in samples taken from female patients (Figure 2C). Overall, male patients had an average NA concentration of 17.09 ng/μl, while the average concentration was 18.66 ng/μl in samples from female patients.

To assess the role of centralized testing, we stratified the clinical specimens into three groups: (I) samples taken by local health authorities (Figure 2D, center panel), (II) the central emergency department (Figure 2D right panel), and (III) the remaining samples (Figure 2D left panel). Swabs taken by the health authority of the city of Essen yielded eluates with an average NA concentration of 17.46 ng/μl. With 24.99 ng/μl NA, the swabs taken at the Central Emergency Department of University Hospital Essen resulted in significant ($p < .001$) higher average NA concentration than all other sample groups. Accordingly, we also observed a difference in the number of samples below the cut-off value. Among the samples provided by the health authorities, only 2.96% (six samples) had a concentration below the negative cut-off of 2.93 ng/μl, and only one sample (1.12%) from the central emergency department was of such

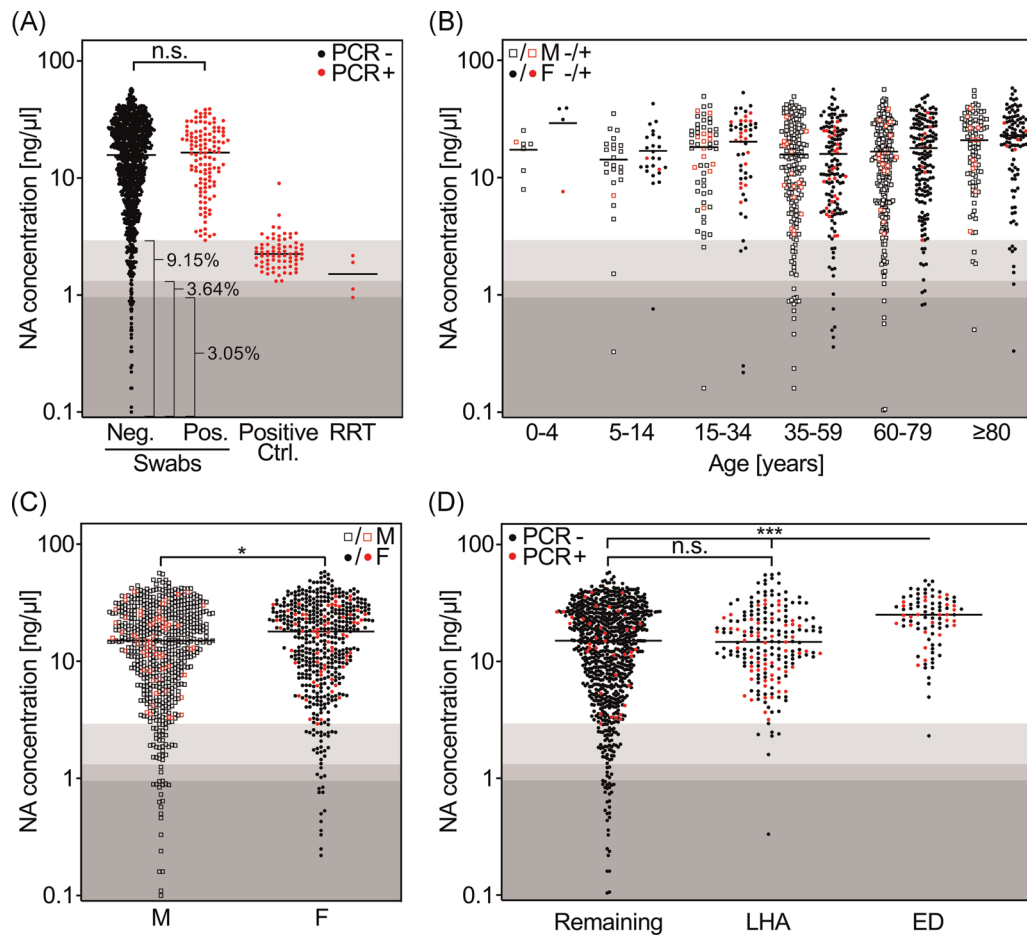


FIGURE 2 Higher quality swabs generated by experienced personnel. Average of duplicates. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) PCR test results are shown as following: negative: black; positive: red. (A) Samples were divided by swabs, internal positive control and round-robin test (RRT) samples. Significance was calculated by unpaired two-tailed *t* test. (B) Swab samples were separated into age groups and sex. (C) Comparison of swab samples acquired from male (M) and female (F) patients. Significance was calculated by unpaired two-tailed *t* test. (D) Samples taken by local health authorities (LHA), the emergency department (ED) and remaining samples were grouped by place of collection. Significance was calculated by unpaired two-tailed *t* test. PCR, polymerase chain reaction

low quality. For comparison, 10.17% (86 samples) of the remaining samples exhibited too low NA concentrations (Figure 2D). Approximately 44% (39 samples) of samples taken at the emergency department were from male patients and local health authorities similarly provided approximately 44% (88 samples) of samples derived from males.

There is an ongoing debate concerning the occurrence and potential reasons for longitudinal sample series derived from one patient that shows intermittent SARS-CoV-2-negative results. Such results have either been attributed to a viral recurrence (see, e.g.,^{31,32}) or inadequate sample quality in a fraction of samples (see, e.g.,³³). Obviously, both explanations are not mutually exclusive. Some intermittent negative samples may have clinical or virological reasons, such as periods of viral replication exclusively in the lower respiratory tract inaccessible for swab sampling or virus recurrence. In contrast, other negative results might simply stem from swabs of insufficient quality or inadequate NA extraction. Between March and June 2020, a few patients who had at least one intermitting negative

SARS CoV-2 RT-qPCR result were tested at University Hospital Essen, eight of whom were examined exemplarily here. All these tests were performed with eluates from swabs. Three to six swabs per patient were collected in 8-31 days after the first sampling. The S and E gene C_t values were measured by RT-qPCR. If previous tests were positive or a SARS-CoV-2 infection was suspected based on clinical or epidemiological circumstances, the local diagnostics department judges samples as SARS-CoV-2-positive if the C_t values are below or equal 42. For easier visualization, the delta between the actual C_t value and the maximum C_t of 42 is depicted. In half of the infection courses (Figure 3A-D), NA concentrations in the SARS-CoV-2-negative samples were only slightly lower than in the adjacent samples tested positive, suggesting that improper swab sampling or NA extraction fail to explain these intermittent negative results. However, in several sample series, an apparent similarity between the slope of SARS-CoV-2 detection and NA concentrations became apparent (Figure 3E-G). In one infection course (Figure 3E), one negative tested sample had a much lower NA concentration than the

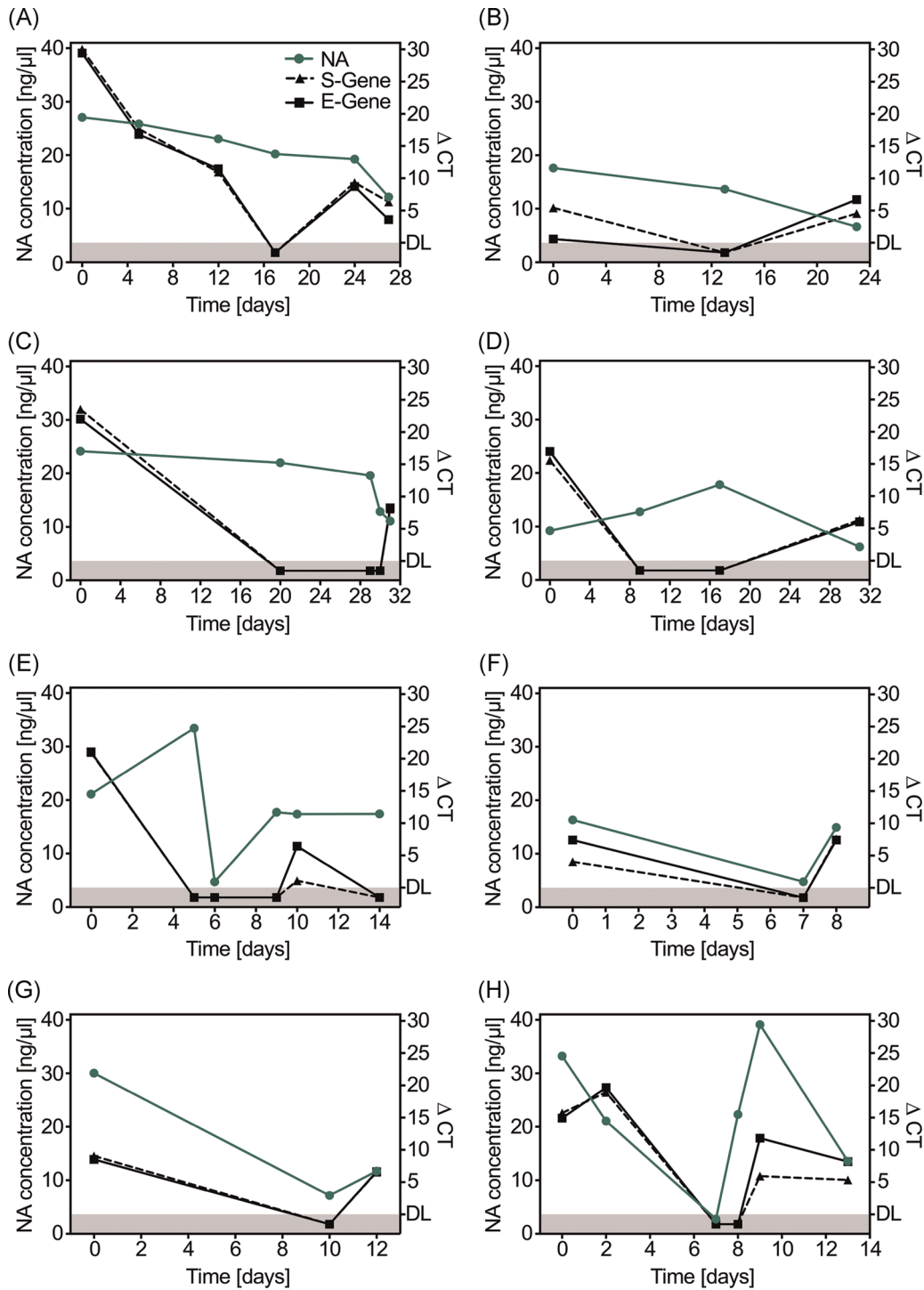


FIGURE 3 Insufficient nucleic acid (NA) concentrations can pretend intermitting negative test results. Eight patient sets (A-H) with intermitting negative tested samples for SARS-CoV-2. Values for NA concentration plotted on left y-axis. An average of duplicates is depicted. The detection limit (DL) was set to a CT value of 42. The delta is plotted on the right y-axis. SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

other samples. Courses G and H both had three samples tested during a period of 8–12 days, with the middle sample being SARS-CoV-2-negative. In both cases, the negative tested eluate had a much lower NA concentration. In one course (Figure 3H), the SARS-CoV-2-negative sample on Day 7 even exhibited a NA concentration

(2.75 ng/μl) below the lower cut-off of 2.93 ng/μl, indicating that it would have been extremely difficult to detect SARS-CoV-2 based on such a low NA concentration in the swab-derived eluate. Thus, the herein described approach allows the identification of samples with NA concentrations insufficient for reliable SARS-CoV-2 diagnostics.

4 | DISCUSSION

To provide quality control for NA eluates used for swab-based SARS-CoV-2 testing by RT-qPCR, we established a protocol applying an NA-specific dye. All reagents and disposables are commercially available and cost-effective (<0.25€ per sample). Devices capable of quantifying the corresponding fluorescence signals are broadly available in diagnostics departments. The test is very fast given that a 96-well-plate with 40 samples, controls, and calibrators can be processed in less than an hour. Thus, the methodology should be immediately applicable to the quality control of clinical specimens used for SARS-CoV-2 diagnostics. Obviously, the test is not limited to SARS-CoV-2 testing and can be utilized to quantify NA concentrations in all sorts of clinical specimens used for diagnostics.

Among clinical specimens, we did not find SARS-CoV-2-positive eluates with NA concentrations below 2.93 ng/μl, suggesting that lower NA concentrations in swab-derived samples impede successful virus detection. Based on this cut-off value, at least 90.85% of the tested swabs contained sufficient material enabling reliable SARS-CoV-2 diagnostic testing by RT-qPCR. In-house positive controls and positive samples from federal round-robin evaluations had much lower NA concentrations. Cut-offs based on these tests would result in even lower boundaries of SARS-CoV-2 detectability in terms of NA concentrations in eluates derived from swab material. Given that the in-house positive control mixture was tenfold diluted with cell culture media and the round-robin test samples were derived from infected cell culture material, we consider applying the highest NA concentration cut-off value established based on positive clinical specimens more reliable. Even with this approach, our data paints a far more favorable picture than previous studies which assumed a false-negative rate of up to 29%.³⁴ Although it is excellent news that greater than 90.85% of swabs contained enough material to enable SARS-CoV-2 recognition, the remaining low-quality swabs and NA elutions make up a vast number of unreliable tests. Given that swab disposables, reagents and procedures as well as the NA extraction devices and kits are used globally, we assume that our findings can be extrapolated to other regions. In this case, 45.75 million tests worldwide may have been performed using inappropriate clinical specimens so far and approximately 366,000 are added daily. Since we observed significantly higher quality swabs from the local health authorities and the emergency department with lower proportions of insufficient samples and higher median NA concentrations, these data highlight an opportunity for optimization. They argue in favor of centralized institutions at which swabs are collected by personnel with continuous routine. Such institutions should be well-connected to the actual test laboratory where the NA extraction and RT-qPCR are performed. Thereby time between sampling and testing is minimized as longer layover times may correlate with degeneration of free NAs and increased chances of bacterial or fungal contaminations, reducing swab quality.³⁵

There is a similar number of laboratory-confirmed cases in both sexes in Germany.³⁶ It has been reported that female patients might be more prone to SARS-CoV-2 infections since the host entry

receptor ACE2 is encoded on the X chromosome.^{37–39} However, upon SARS-CoV-2 acquisition, the course of COVID-19 is known to be more severe in men than in women and sex hormones are considered to play a role in these sex-specific differences.^{40,41} Other risk factors, such as obesity and smoking being more prevalent in men may also contribute. Further experiments will be necessary to explain the significant ($p = .03$) sex-related difference concerning NA concentrations in swab-based eluates between male and female patients herein described—to our knowledge—for the first time.

If NA concentrations of the eluate are not evaluated, a negative SARS-CoV-2 test can result either from the absence of an infection or the lack of sufficient test material. The fact that a considerable proportion (~10%) of eluates showed such low NA concentrations strongly advocates for implementing quality control measures for example by the herein described methodology. Otherwise, false-negative results may occur, which may postpone or prevent adequate antiviral therapy. In epidemiological surveillance programs, false-negative results can prevent the rapid identification of index cases and super-spreaders with catastrophic consequences for society.

Since NA quantification is fast and cost-effective, it can be used to save expensive and scarce RT-qPCR reagents and disposables. Especially during periods of limited supply and availability, reagents, disposables and sample slots in diagnostic devices should not be wasted for eluates with insufficient NA concentrations impeding virus recognition. This issue is particularly exemplified in a peculiar clinical constellation: there are numerous reports of patients with intermittent negative test results for SARS-CoV-2.^{19,42–44} Possible explanations for this are improper testing and changes in the viral distribution pattern. The latter has been described for SARS-CoV and MERS-CoV.^{45,46} Additionally, there have been a few reports of genuine recurrent SARS-CoV-2 infections.^{47,48} Our study of eight intermittently negative patients shows that NA quantification may help to identify intermittent false-negative samples. All of the patients showed comparatively high C_t values in the recurrently positive samples indicating low viral loads at the time of testing. Accordingly, the C_t values of all recurrently positive samples were above 30, which is in line with findings made by the Korea CDC on intermittent negative patients.¹⁹ It goes without saying that it is particularly important to obtain high-quality swabs providing sufficient NA for virus recognition in borderline positive patients with reduced viral loads.

Taken together, the herein described fluorescence-based NA quantification approach is immediately applicable to evaluate swab qualities, optimize sampling strategies, identify patient-specific differences, and explain some peculiar test results, such as intermittent negative samples.

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CONFLICT OF INTERESTS

The authors declare no conflict of interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTIONS

Conceptualization: Vu Thuy Khanh Le-Trilling, Ulf Dittmer, and Mirko Trilling. Methodology and data curation: Robin L. Kligen and Benjamin Katschinsk. Investigation, Robin L. Kligen, Benjamin Katschinski, and Olympia E. Anastasiou. Resources: R. Stefan Ross. Writing—original draft preparation: Robin L. Kligen, Benjamin Katschinsk, and Mirko Trilling. Writing—review and editing: all authors. Supervision: Mirko Trilling and Vu Thuy Khanh Le-Trilling. Project administration and funding acquisition: Mirko Trilling. All authors have read and agreed to the published version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

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

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