requiring different verification strategies, supply chain issues, the need for high- and low-throughput molecular testing with unrealistic demands on turnaround times, the need to test symptomatic and asymptomatic individuals, and the endless demands on staff affected our testing capacity.

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DNA Cross-Reactivity of the CDC-Specified SARS-CoV-2 Specimen Control Leads to Potential for False Negatives and Underreporting of Viral Infection

To the Editor:

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) has been widely used to detect SARS-CoV-2 viral RNA genomes from upper respiratory samples. During the first months of the COVID-19 pandemic, testing focused on patients who were presumed positive in the context of triage and epidemiological monitoring. After the first wave of infection, molecular testing has expanded to a population with a high true-negative rate—clearing asymptomatic individuals for return to work, school, and society based on absence of detectable virus. Minimizing analytical false negatives for COVID-19 testing is a public health imperative.

Multiple RT-qPCR test designs for detecting SARS-CoV-2 have been developed and given emergency use authorization by the US Food and Drug Administration (FDA). As of July 2020, nearly half of the FDA-authorized designs use qPCR primers and probes specified by the US Centers for Disease Control and Prevention (CDC). The CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel is comprised of 3 qPCR assays: N1 and N2 generate SARS-CoV-2-specific amplicons from reverse transcribed viral RNA; RP is a human specimen and extraction control that targets a single exon within the human *RPP30* gene. Failure of this control is intended to indicate potential loss of RNA or RNA degradation (1). In practice, this design generates a misleading control-positive signal in scenarios that preclude RNA virus detection.

The CDC RP control is capable of detecting reverse transcribed RNA; it also detects human genomic DNA. Indeed, the CDC uses the same control design for RT-qPCR and qPCR panels targeting viral respiratory pathogens with RNA and DNA genomes, respectively (personal communication with CDC). When human DNA is present, intact RNA and reverse transcription are unnecessary to generate a positive specimen and extraction control signal.

Single-digit copies of genomic DNA are sufficient to generate a strong control signal using the CDC design (data not shown). DNA is copurified by solid phase and liquid–liquid extraction procedures used for isolation of RNA from clinical specimens. qPCR-only (no-RT) reanalysis of RNA samples extracted from COVID-19 case nasopharyngeal swabs yielded strong control signals from all specimens tested (data not shown). More worryingly, DNA cross-reactivity leads to analytical false negatives from true-positive patient samples where RNA has been degraded (Fig. 1).

Pooling multiple samples prior to analysis is being used to increase throughput and reduce testing cost (2–4). The problems caused by a DNA-reactive control are magnified by pooling: one RNase-containing sample can render an entire pool virus RNA negative, while a few cells' worth of DNA from a single patient are sufficient to generate a specimen

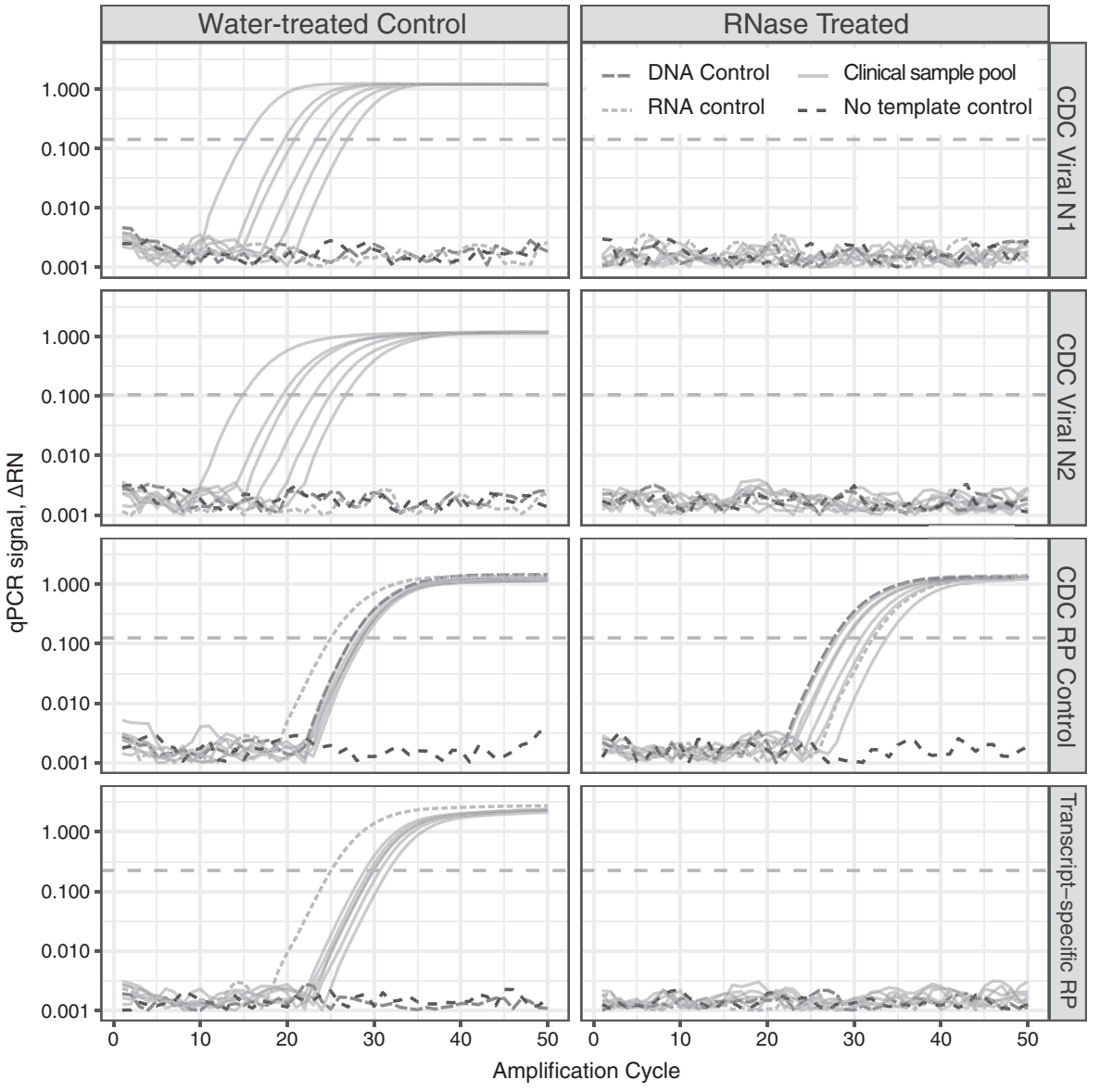

Fig. 1. The de facto standard COVID-19 RT-qPCR test design includes a specimen and extraction control that is DNA cross-reactive and generates analytical false-negative results following loss of RNA integrity. Purified nucleic acid samples from COVID-19 positive cases were pooled, aliquoted, and treated with either nuclease-free water or ribonuclease A (RNase). Commercial human RNA, DNA, and no-template controls were treated in parallel. Samples were analyzed by RT-qPCR with Viral One-step master mix using the CDC-specified Viral N1, Viral N2, and human specimen and extraction control (RP) assays, plus a transcript-specific control targeting spliced exons 1–3 of *RPP30* (Hs.PT.58.2854947, idtdna.com). Strong viral and specimen control signals are generated from each infected-patient pool after mock treatment (water, left). Purified DNA is sufficient template for the CDC RP control. Loss of RNA integrity precludes detection of viral genomes, while the cross-reactive specimen control continues to generate strong signal from copurified DNA (RNase, right). A transcript-specific RP control properly reflects the loss of sample integrity and returns no signal from samples containing degraded RNA.

and extraction control signal. A DNA-reactive control opens the door to silent assay failures and false-negative reporting of individuals who were COVID-19 positive from whom virus was successfully collected and whose samples were intact prior to pooling with dominant negative samples.

The absence of viral signal is insufficient for clinical interpretation. Controls must demonstrate that the test worked as intended and would have found virus had it been present. The current goal of testing is not just to find needles in haystacks—it is to conclusively state that individual haystacks contained no needles.

This widely used design has high analytical sensitivity for detecting the SARS-CoV-2 virus, but incorrectly reports "assay success, no virus found" when faced with degraded specimens. A specimen and extraction control that specifically detects human RNA (Fig. 1) eliminates this preventable class of false-negative results and can improve negative predictive value. A redesigned control will properly return "don't know" instead of incorrectly reporting "no." This distinction enables focused retesting and conservative clinical management rather than prematurely giving an all-clear.

Nonstandard Abbreviations: RNA, ribonucleic acid; SARS-CoV-2, severe acute respiratory syndrome-associated coronavirus 2; cDNA, complementary DNA; COVID-19, Coronavirus disease 2019; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RNase, ribonuclease; CDC, US Centers for Disease Control and Prevention; DNA, deoxyribonucleic acid; EUA, emergency use authorization; FDA, US Food and Drug Administration; N1, N2, CDC SARS-CoV-2viral detection amplicons 1 and 2 (CDC abbreviation); RP, *RPP30* specimen and extraction control (CDC abbreviation)

Human Genes: *RPP30* ribonuclease P/MRP subunit p30 (deprecated name *TSG15*).

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Effect of Pneumatic Tube System Transport on Cell-Free DNA

To the Editor:

In patients with cancer, a small fraction of the cell-free DNA (cfDNA) may contain tumor-derived