Title: Patient DNA cross-reactivity of CDC SARS-nCoV2 extraction control leads to potential false negative results

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Abstract:

Detecting RNA viruses such as SARS-nCoV2 requires careful handling of inherently labile RNA. Molecular tests incorporate controls to verify the presence of intact RNA and ensure that all steps of an assay have succeeded. The Centers for Disease Control and Prevention (CDC)-specified extraction control recognizes both reverse-transcribed RNA *and* human genomic DNA. Human DNA copurified from nasopharyngeal swabs by multiple clinically-used RNA extraction approaches, is sufficient for a strong "extraction control positive" signal using the CDC design, creating the potential for false-negative results. Moreover, DNA cross-reactivity precludes control of RNA integrity in real-world samples. A properly designed control that monitors sample collection, extraction, reverse transcription, and qPCR processes is essential and demonstrated here. This control can be immediately implemented in the CDC testing workflow.

One Sentence Summary:

A widely-used COVID-19 diagnostic is misdesigned and can report "assay okay, virus not detected" from virus-containing samples – but it's fixable

Main Text:

Molecular diagnostic testing has become (socially-distanced) dinner table conversation during the COVID-19 pandemic. Reverse-transcription coupled quantitative polymerase chain reaction (RT-qPCR) has been widely deployed to measure viral RNA genomes present in patient samples. SARS-nCoV2 RT-qPCR assays can detect virus from early stages of infection *(1)*. Measuring viral RNA informs as to presence of virus at the time of sampling and can be used as a measure of potential infectivity. Measuring viral RNA complements serological testing that can only indicate past infection. During the first months of the COVID-19 pandemic, testing has focused on enumerating positive patients in the context of triage and epidemiological monitoring. As the world begins to reopen, the focus of testing will shift to *clearing* asymptomatic individuals as "no detectable virus present."

The US Centers for Disease Control and Prevention (CDC) have specified and given emergency use authorization (EUA) for a SARS-nCoV2 molecular diagnostic used to detect viral RNA in clinical samples *(2)*. As shown here, this assay uses a poorly-designed specimen extraction control that does not perform as intended on real-world samples. This control falsely reports on specimen and process integrity. False negatives are generated when assays report "assay functioned correctly and no virus was detected" from truly positive patients. False negatives undermine clinical sensitivity with varying effects depending on disease prevalence, mode of transmission, and context. Patients would be incensed by a preventable false-positive diagnosis

putting them into unnecessary quarantine. The public should be incensed by an assay whose preventable false-negative diagnoses unwittingly allow virus-positive patients back into society.

The 2019-nCoV Real-Time RT-PCR Diagnostic Panel is a CDC-designed and validated COVID-19 RT-qPCR test containing three qPCR primer/probe sets. N1 and N2 generate SARS-nCoV2-specific amplicons to detect viral RNA. The third, RP, targets the human *RPP30* gene and is an "extraction control" intended to inform about successful sample collection, RNA extraction, and reverse transcription *(3)*.

The CDC design is widely used and can identify SARS-nCoV2-positive patients. Early testing during the COVID-19 pandemic has focused on triage of presumed-positive patients and testing of the symptomatic public with a high true-positive rate. A shift in focus to ensuring that individuals are virus-free makes reducing false-negative results a public health imperative. The CDC design is fundamentally flawed and has the potential to return dangerous false negatives (Fig. 1A). A solution to this problem is straightforward: a transcript-specific *RPP30* control correctly reports on successful sample collection, extraction, and reverse transcription. This improved design is of immediate use, can increase clinical sensitivity of testing, and will reduce preventable false-negative SARS-nCoV2 results.

The CDC-specified RP extraction control design generates identical amplicons from reverse transcribed RNA and human genomic DNA. Megablast *(4)* search of the RP forward, reverse, and probe sequences against the human refseq_rna transcript database identifies perfect matches, as intended, within the *RPP30* mRNA. The CDC primer/probe design generates an amplicon that is entirely contained within the first exon, and therefore generates an identical amplicon from the *RPP30* genomic locus (Fig. 1B). As designed, the CDC extraction control generates a positive signal in reactions containing intact, reverse-transcribed RNA. Unfortunately, the control primers and probe also generate a positive signal from human genomic DNA, resulting in potential false negatives (Figs. 1A and S1).

Genomic DNA is co-purified during work-up of clinical RNA specimens in quantities sufficient to generate strong positive signals for the CDC-specified extraction control. SARS-nCoV2-positive clinical samples were analyzed using the CDC extraction control probe in qPCR reactions lacking reverse transcriptase to measure only DNA. "Purified RNA" samples from COVID-19 cases were obtained from a CAP/CLIA certified university hospital molecular pathology laboratory. Samples had been processed using either solid phase extraction (Qiagen) or low pH organic extraction (Trizol). Both methods are permitted under current Emergency Use Authorization (EUA) for RNA isolation. In reality, both methods co-purify significant amounts of DNA *(5)*. All of the clinical samples tested generated unambiguous "extraction control positive" signals in the absence of reverse transcription (Fig. 2A). This result demonstrates that the CDC-designed RP probe does not require reverse transcription to generate a "successful extraction" signal from clinical samples, invalidating one of its claimed control functions *(6)*.

Single-digit copies of genomic DNA are sufficient to generate a positive control signal using the CDC-designed assay. Driven by concern about the control probe signals observed in PCR-only analysis, I determined the quantity of genomic DNA required to generate an assay-positive control signal. Commercially supplied, pooled human RNA-free DNA was serially diluted across a several orders of magnitude and analyzed by the same one-step RT-qPCR used for clinical samples (Fig. 2B). Extrapolating from pure-DNA samples, the "positive control signal"

cutoff would easily be achieved by samples containing a single copy of the genome copy per reaction *(7)*. Reverse transcription is required to convert viral RNA into cDNA suitable for qPCR detection, but is unnecessary to amplify co-purifying genomic DNA. Since the CDC design generates an identical "extraction control" signal from both DNA and cDNA, it is unable to serve as a reverse transcription control on clinical samples.

Due to the presence of co-purifying genomic DNA, loss of RNA integrity in clinical samples leads to false-negative results using the CDC-specified control. RNA, including the genome of the SARS-nCoV2 virus, is intrinsically more labile than DNA. RNA-degrading enzymes (RNases) rapidly degrade RNA while leaving DNA intact. Laboratory procedures that manipulate RNA, including methods specified in the CDC EUA, require careful sample, reagent, and consumable handling to avoid introduction of ubiquitous environmental or personnel-derived RNases. Exposure to RNase causes SARS-nCoV2 virus-positive clinical samples to return an "extraction control, virus negative" result using the CDC-design (Fig. 3). A transcript-specific design correctly controls for RNA integrity and successful reverse transcription, serving all intended roles of the human specimen / extraction control (Figs. 3, S1, and S2 and Supplementary Text).

Testing for RNA viruses is dependent on reverse transcription of RNA to cDNA. Once reverse transcribed, cDNA can serve as a template for PCR. So, too, can other DNAs present in the sample, including patient genomic DNA. Assays can be designed to selectively detect mature, spliced RNA by targeting sequences created during mRNA splicing and that are entirely absent from genomic DNA (Figs. 3, S1-3). Unfortunately, the current CDC control is not transcript-selective, and readily uses both RNA and DNA as a template. This design choice makes the current control probe incapable of performing another intended role, monitoring of RNA integrity *(3)*.

Methods for nucleic acid extraction generally co-purify RNA and DNA. Whether using solid phase extraction or liquid-liquid partitioning, lab automation systems or pipetting by hand, co-purification of patient DNA is a practical reality. All clinical samples tested in this study contained genomic DNA in quantities sufficient to generate a clear-positive "extraction success" result using the CDC assay (*8)*.

The CDC specifies using a once-per-plate control using *in vitro* transcribed RNA, purified virus, or pooled patient samples. This approach does not inform about the suitability or performance of individual specimens. Degradation of sample RNAs or failure of reverse transcription on a well-by-well basis will generate a false negative result even for patients from whom virus was successfully collected and present at the time of sample extraction.

Properly-designed extraction and reverse transcription controls are essential, not for detecting SARS-nCoV2, but for determining when an a test result is invalid. The unprecedented scale of molecular testing undertaken during the COVID-19 pandemic has required that labs rapidly adopt this CDC-specified, among other, new assays. Labs that have been accustomed to automated walk-up systems are now performing manual sample handling and reaction setup one pipet tip at a time. The CDC design detects SARS-nCoV2. It must, and can, be redesigned to also affirmatively report when something has gone wrong.

Controls for specimen integrity and success of all reaction steps are critical to robust assay design. The currently-specified CDC control achieves neither. A number of FDA-approved SARS-nCoV2 molecular assays use synthetic RNA spike-ins added at the time of extraction.

This approach controls for post-extraction sample integrity and reverse transcription, but does not inform about sufficiency of collected patient samples *(9)*. The intent of the CDC's extraction control approach is a good one, monitoring (1) sufficient human specimen collection, (2) maintenance of sample integrity during processing, and (3) successful reverse transcription and qPCR. An extraction control that specifically detects human RNA and is insensitive to genomic DNA is both necessary and sufficient to perform these three control functions.

The flawed CDC control probe is used in over 40% of the COVID-19 FDA-authorized protocols listed as of May 13, 2020 *(10)*. 31 million assays worth of CDC-design reagents have been produced and shipped by a single vendor as of April 22, 2020 *(11)*. This CDC-designed COVID-19 assay is unquestionably popular, but we must not confuse popularity and official approval with being correct. The scope of this potential problem is large, though its extent is unknowable: the publicly-unavailable probe sequences used in many test designs preclude critical review. Even fully disclosed sequences do not guarantee rapid discovery of errors. This poorly-designed CDC extraction control probe has been included in panel designs targeting RNA viruses for over a decade *(12)*.

The current CDC algorithm states that molecular testing "…must be combined with clinical observations, patient history, and epidemiological information"*(2)*. In the context of drive-up testing and potential nationwide return-to-work-clearance testing, clinical observations will be necessarily limited and often impersonal in scope. Absence of data and data of absence must be carefully distinguished. A proper control does not provide definitive diagnostic results from a flawed specimen. It will, however, fail-safe, flag a result as invalid, and call attention to problem samples to enable focused re-testing or changes in clinical management.

In the interest of public health, assays must generate as many valid results as possible. This requires simultaneously maximizing identification of true positives *and* minimizing false negatives. Virus-shedding patients must not be given an all-clear test result due to an improperly designed that can be readily fixed. While perfect diagnostic tests are unachievable, assays should be designed and updated to strive for maximum specificity. The cost of incorrectly clearing SARS-nCoV2-positive patients is incalculably large. Implementing a redesigned control that performs as intended amounts to pennies per test. A new primer / probe set, such as the one described here, is a drop-in replacement that can be manufactured rapidly and at scale. It is imperative that the CDC take action to update its clinical algorithms and specify a re-designed transcript-specific extraction control that performs all functions as originally intended.

References and Notes:

1. R. Wölfel *et al.*, Virological assessment of hospitalized patients with COVID-2019. *Nature*, (2020).
2. U.S Centers for Disease Control and Prevention, (CDC), "CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel Instructions for Use" (Publication CDC-006-00019, Revision: 03, Centers for Disease Control and Prevention: Division of Viral Diseases, 30 March 2020; https://www.fda.gov/media/134922/download).
3. As per the CDC EUA, failure to observe the RP control can reflect: (1) Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation. (2)

Absence of sufficient human cellular material due to poor collection or loss of specimen integrity. (3) Improper assay setup and execution, or (4) Reagent or equipment malfunction.

4. M. Johnson *et al.*, NCBI BLAST: a better web interface. *Nucleic Acids Res* **36**, W5-9 (2008).
5. The amount of co-purified DNA is lower for Trizol-purified samples despite use of a larger fraction of the sample. This may reflect an even greater RNA:DNA co-purification ratio but may also reflect evolving clinical skills in patient sample collection during COVID-19 testing.
6. The CDC EUA states that the extraction control is considered positive / indicative of a successful extraction, where control probe Ct < 40 cycles using a 7500FASTDx instrument (see Supplementary Text).
7. Multiple vendors of purified "reference" DNA were used with similar results. Promega Human Male control DNA is available in relatively large lot-controlled batches, validated, is widely cited, and is used in all figures shown here.
8. Nasopharyngeal (NP) swabs in transport media are a particularly sanguineous mixture that are frequently received with co-collected patient cells and tissue. While there may be more patient DNA in NP-derived specimens than other collection types, human genomic DNA is routinely intentionally collected from saliva, sputum, and other COVID-19 relevant anatomic sites.
9. A properly-processed blank swab or empty vial of VTM will generate, as intended, a spike-in positive signal.
10. U.S. Food and Drug Administration, (FDA), "Coronavirus Disease 2019 (COVID-19) Emergency Use Authorizations for Medical Devices: High Complexity Molecular-Based Laboratory Developed Tests" (Appendix A Table, May 2020; https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd).
11. Integrated DNA Technologies, (IDT), "SARS-CoV-2 research reagents"(https://www.idtdna.com/pages/landing/coronavirus-research-reagents).
12. U.S Centers for Disease Control and Prevention, (CDC), "CDC protocol of realtime RTPCR for influenza A(H1N1)" (Centers for Disease Control and Prevention: World Health Organization Collaborating Centre for influenza, 6 October 2009; https://www.who.int/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf).
13. Qiagen GmbH, "QIAamp® DSP Viral RNA Mini Kit Handbook" (Publication Reference 61904, Version: 01, QIAGEN GmbH, March 2012; https://www.qiagen.com/us/resources/resourcedetail?id=46638e95-df58-4874-9015-732e75587524&lang=en).

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Supplementary Materials:

Materials and Methods

Supplementary Discussion

Figures S1-S3

Table S1

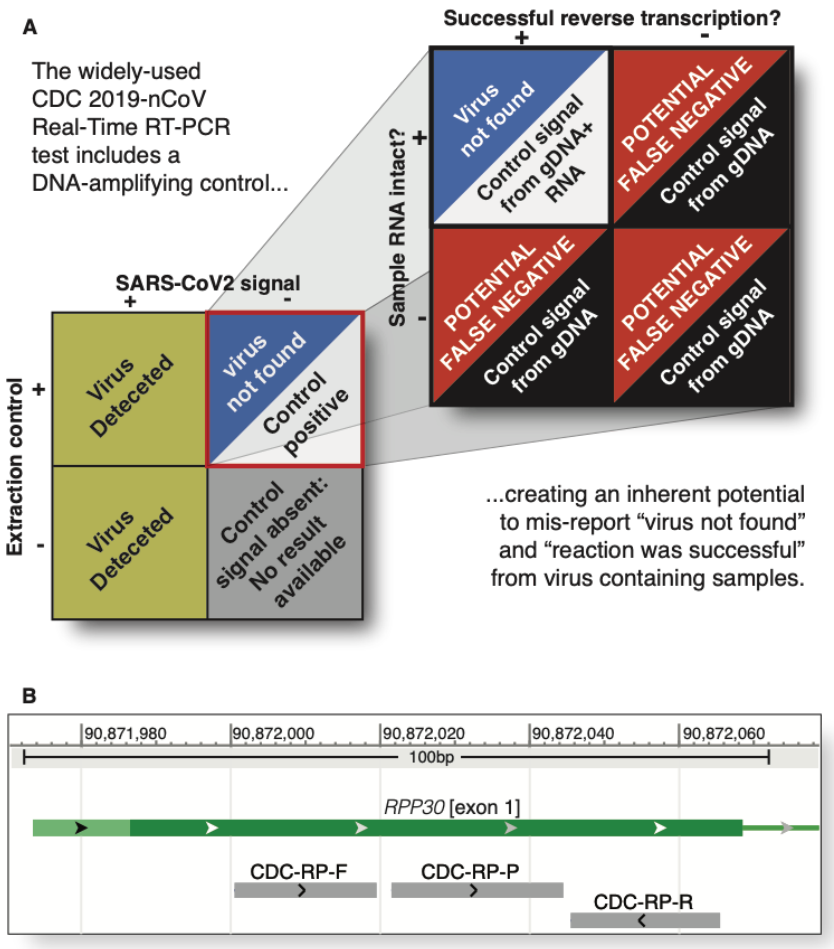

Fig. 1. A. The CDC-specified SARS-nCoV2 qPCR assay and associated clinical algorithm are indended to inform for presence of virus, and if no virus is found, report on a reaction that could have identified virus – but did not. Signal from virus-targeting RT-qPCRs is sufficient to report "Positive 2019-nCoV". An "invalid" report is returned where all three probes were negative, reflecting a problem in sample collection, workup, or qPCR. When the viral probes are negative and a human *RPP30*-targeting control returns a positive signal, clinical interpretation is "2019-nCoV not detected." Patients who are truly virus negative will fall into this category. So will patients for whom any of several possible sample collection, handling, or analysis problems have occurred, due to the cross-reactivity of the control probe for patient genomic DNA. **B.** The CDC-specified extraction control primers and probe recognize a sequence that is identical in both reverse transcribed RNA and genomic DNA. Megablast search of the human refseq_dna database identifies perfect matches for the CDC- control primers, RP-F and RP-R, and probe, RP-P. This design amplifies and detects a 65 base pair amplicon located entirely within exon 1 of the *RPP30* gene. Specific detection of reverse-transcribed RNAs can be accomplished by design of an exon-exon junction spanning amplicon and, optionally, probe (Supplemental Figure 3).

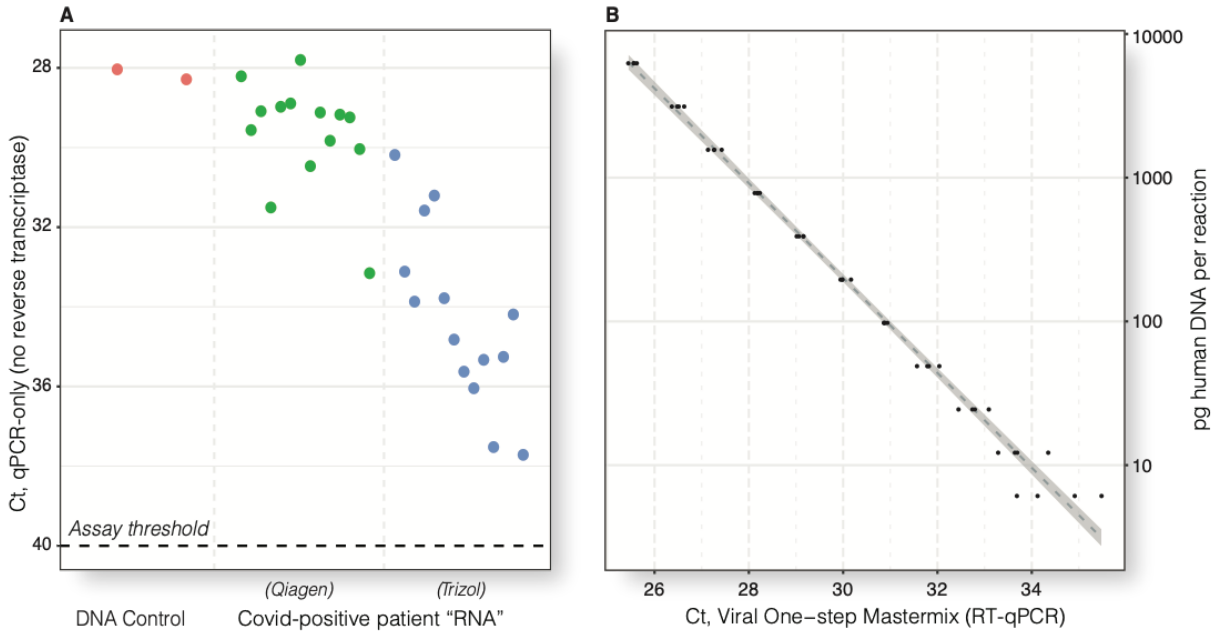

Fig. 2. Genomic DNA, co-purified during RNA extraction of clinical COVID-19 samples, is sufficient to generate a positive signal using the CDC extraction control. **A.** qPCR (without reverse transcription) was performed using the CDC extraction control (RP) probe. Raw Ct values are shown. A total of 28 representative COVID-19 positive clinical samples were tested, including fourteen purified by automated silica spin-column (Qiagen) and fourteen by manual organic extractions (Trizol, Materials and Methods). All samples from both methods generated extraction control positive signals well above the CDC assay threshold (40 cycles, horizontal dashed line). Significantly more genomic DNA was present in samples purified by silica spin-columns compared to organic extraction (mean Ct of 29.6 and 34.3, respectively, $p = 1.21 \times 10^{-6}$, unpaired *t*). Co-purification of DNA is to be expected based on the mechanisms of purification used, and is openly caveated in vendor literature *(13)*. Per-plate process controls contained 8.6 ng of genomic DNA, equivalent to ~1250 diploid cells. **B.** Single-cell equivalents of purified genomic DNA, absent RNA, are sufficient to generate an "extraction control positive" signal using the CDC-RP probe. RT-qPCR was performed using Viral One-Step Mastermix (Materials and Methods) on 2-fold serial dilutions of a commercially-supplied reference human DNA. Control-positive signals are generated from dilution equivalent to two haploid copies of gDNA per reaction (6.125pg), despite increase in Ct variability at extreme dilutions.

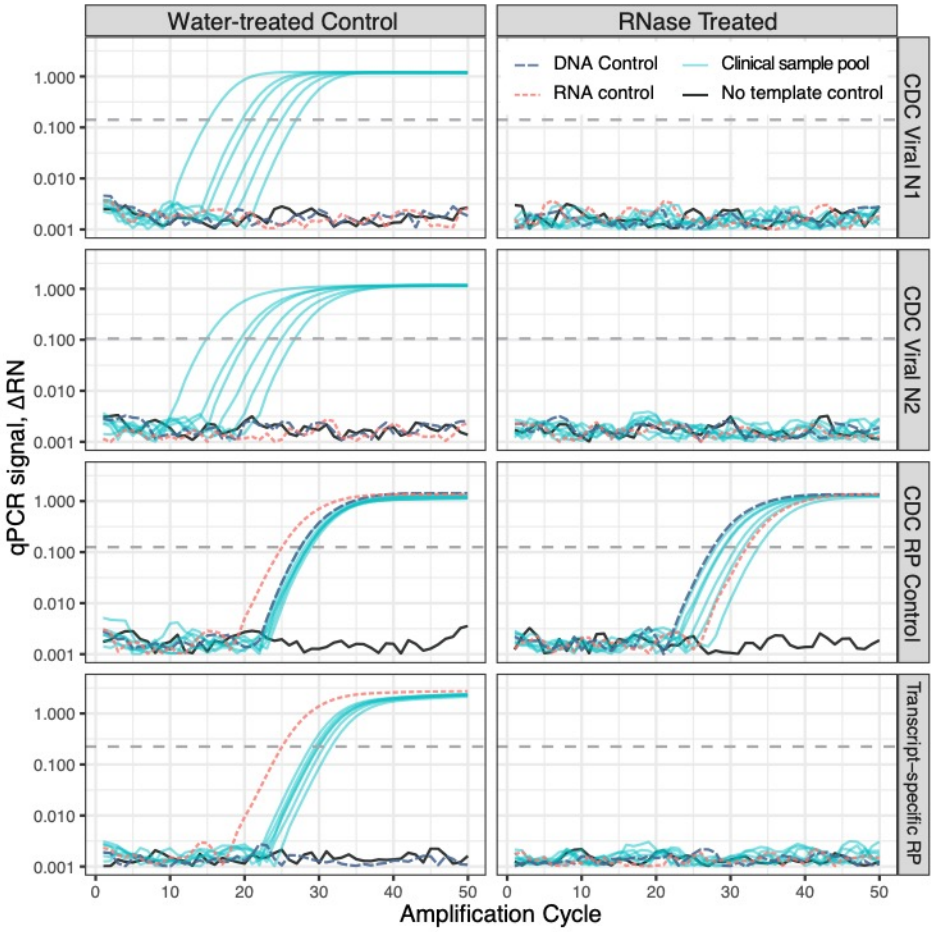

Fig. 3. CDC-specified testing of SARS-nCoV2-positive clinical specimens return a false negative following exposure to ribonuclease. Reverse-transcriptase RT-qPCR data obtained using Viral One-step mastermix are shown as $\log_{10}$(normalized fluorescence) as a function of thermal cycle (Materials and Methods). Extracted nucleic acid samples, used in Figure 2, were pooled, divided into aliquots, and treated with either nuclease-free water or Ribonuclease A (RNase A). Commercial RNA, DNA, and no-template controls (NTC) were treated in parallel. Each treated sample was used as input for RT-qPCR reactions using the CDC-designed RP control, Viral N1, Viral N2 primer/probe sets or a transcript-specific control targeting exons 1-3 of *RPP30*. True positive viral and control signals are observed in all six pools after sham treatment. False negatives are returned from all clinical samples following degradation of RNA. The transcript-specific RP control accurately informs as to loss of sample integrity, which would flag affected samples as "invalid" and necessitate re-testing. Horizontal dashed lines reflect software-generated Ct thresholds for each primer/probe set.