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White Paper

Detection of SARS-CoV-2 Armored RNA in Global Interlaboratory Harmonization Study

In the COVID-19 pandemic, researchers needed reliable access to control material for developing, validating, and running diagnostic tests. A study run at 14 labs around the world shows that bacteriophage-protected (Armored) synthetic controls, which can be more easily distributed at the start of an outbreak, perform comparably to inactive virus.

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Abstract

At the start of 2020, a global effort began to respond to the COVID-19 pandemic by developing molecular tests that could accurately and rapidly diagnose this emerging disease. A critical component was a control to harmonize the results of the myriad tests being created. In order to address this urgent need, a Coronavirus Standards Working Group was formed in March 2020 to provide recommended infrastructure for COVID-19 testing and ensure reliability of test results. This international consortium was convened by the Joint Initiative for Metrology in Biology at Stanford University and included academic, government, private, and nonprofit organizations across a variety of disciplines. The working group systematically considered different aspects of the measurement process, including standards and controls, and their impact on various stages of the testing process. This effort included a study planned by the consortium and executed globally by independent laboratories to assess multiple sources and types of molecular controls. The study involved 14 laboratories worldwide that were provided SARS-CoV-2 RNA control material from eight vendors, one of which was Asuragen. Additionally, World Health Organization International Standards (WHO-IS) were prepared for each laboratory to use as calibrators. Here we describe Asuragen's SARS-CoV-2 Panel Control performance against WHO-IS across the different laboratories, assays, and platforms used.

Introduction

The global pandemic was declared in March 2020 by the World Health Organization and the Centers for Disease Control to raise awareness and begin combating spread of the deadly SARS-CoV-2 virus causing COVID-19. Even before this declaration was made, laboratories around the world had scrambled to sequence the viral genome and develop diagnostic tests specific to this strain of the virus[1]. Molecular tests were chosen initially over antibody tests for their ability to detect the virus directly at symptom onset and before antibody load was sufficiently high to detect. Additionally, molecular tests provided higher sensitivity and specificity to allow a definitive diagnosis using more easily attained synthetic sequences as controls (FIGURE 1). Many of the first iterations of molecular tests were problematic in terms of specificity and/or sensitivity[2], a result of accelerated development to meet the exponentially growing demand for testing as cases soared. Complications also arose due to limited supply of transport medium and positive patient samples needed to validate tests. Though SARS-CoV-2 shares homology with viral genomes of other SARS strains, limitations in sequence availability of the new strain and associated variants created a lack of consensus for test developers.

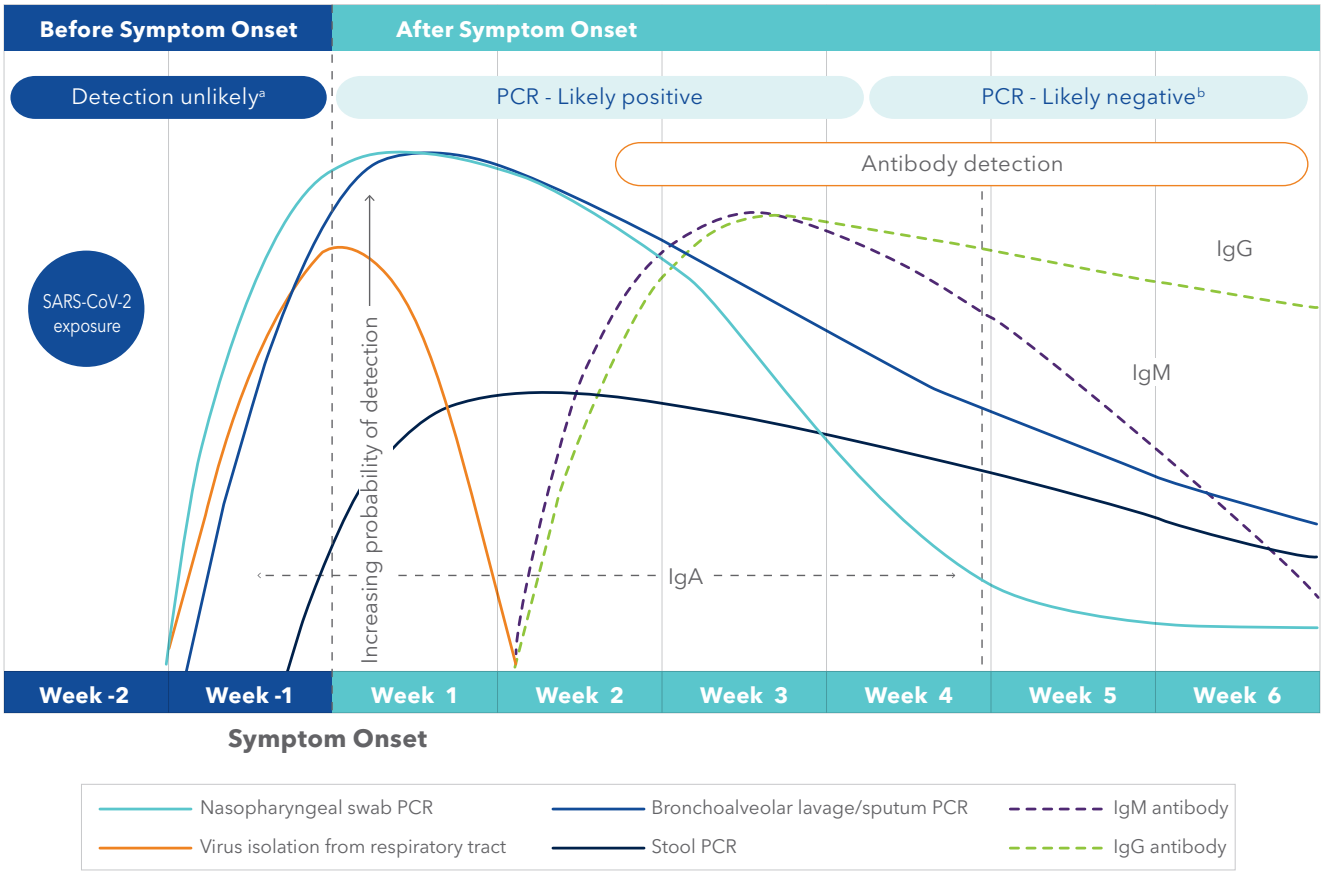
During assay development, there are several factors to optimize beyond definition of a consensus sequence to target. Some of these include selection of optimal nucleic acid extraction methods, controlling for cross-contamination, evaluating sensitivity and specificity of chosen primers and/or probes on widely available detection platforms, and securing sufficient control material to conduct these optimization studies[3].

The four most commonly used types of viral control material are inactivated virus, recombinant viral-like particles (VLPs), recombinant bacteriophage, and naked synthetic RNAs. Typically, they are used as either positive run controls or exogenous internal controls. Each has unique advantages depending on the stage of the assay being optimized[4,5]. Although having greatest utility for assessing real-world performance, inactivated virus is least available at the beginning of an outbreak and poses a significant health risk if not handled properly. VLPs and recombinant bacteriophage RNAs have the advantage of mimicking a virus without the risk of infectivity. Recombinant bacteriophages and naked synthetic RNAs provide the greatest flexibility in tailoring the viral genetic sequence to include specific regions of interest.

Through the effort of the newly formed Coronavirus Standards Working Group, which aims to provide guidance to the scientific community in monitoring and controlling for high variability in testing during the initial phases of the pandemic, a plan was devised to initiate a Molecular RNA Harmonization Study to ensure test accuracy. This was a global collaborative effort convened by the Joint Initiative for Metrology in Biology at Stanford University and included academic, government, private, and nonprofit organizations across a variety of testing-related disciplines.

Asuragen's Armored RNA Quant SARS-CoV-2 Control was developed and released in March 2020 to support efforts to bring more diagnostic tests to the market. Armored controls consist of synthetic, targeted genetic material packaged within a bacteriophage capsid. This provides needed protection from degradation and makes it ideally suited to use as a full process or run control. The Armored RNA Quant SARS-CoV-2 Panel control used in this study contains the nucleocapsid (N1/N2), envelope (E), RNA-dependent RNA polymerase (RdRp), and open reading frame 1 (ORF1) regions of the SARS-CoV-2 genome.

Altogether eight control manufacturers including Asuragen submitted material to Stanford to be distributed to 14 laboratories for routine testing in replicate alongside a WHO-IS (World Health Organization International Standards) standard curve for extrapolation of quantitative values. The controls showed comparable levels of expected quantities, albeit with some limitations, across a variety of SARS-CoV-2 genomic regions targeted by multiple platforms, chemistries, laboratory-developed tests, and tests given Emergency Use Authorization by the FDA.



[a]Detection only occurs if patients are followed up proactively from the time of exposure.
[b]More likely to register a negative than a positive result by PCR of a nasopharyngeal swab.

FIGURE 1. Estimated variation over time in diagnostic tests for detection of SARS-CoV-2 infection. (Sethuraman, *et al.*, JAMA. 2020;323(22):2249-2251)

Materials and Methods

Curated Control Samples

Eight vendors provided control material for this study. Controls were sent in quadruplicate aliquots to 14 laboratories worldwide (FIGURE 2). Each vendor provided a concentration that had been derived from different methods. These nominal concentrations ranged from 5E+03 copies/mL to 2E+10 copies/mL. A comprehensive standard operating procedure was provided to the laboratories to ensure consistent handling of the controls provided. In addition to the eight vendor controls, the National Institute for Biological Standards and Controls provided the high-titer inactivated viral WHO-IS reference standard; each laboratory was instructed to construct an eight-point standard curve from 1E+08 to 0 copies/mL for normalization. According to the standard operating procedure, the Asuragen control was to be diluted 1:100 twice in provided diluent; all other controls were run neat. Upon testing by their own internally developed and validated assays, labs sent results back to Stanford researchers for compilation and dissemination.

Data Generation at Laboratories

The RNA extraction methods used by the 14 laboratories varied from column-based viral RNA extraction kits to magnetic bead-based extraction kits to all-in-one systems that did not require extraction ahead of processing. Platform chemistries included probe-based detection methods on digital PCR using Bio-Rad systems (QX200, T100, C1000) and real-time PCR using instruments from Roche (Cobas6800), Fluxergy, Abbott (Alina m), ThermoFisher (7500Fast and QuantStudio), and Bio-Rad (CX384). Five out of the 14 laboratories used digital PCR, while the rest used real-time PCR. Of the nine laboratories using real-time PCR, three involved platforms that required no extraction prior to loading the sample on the instrument.

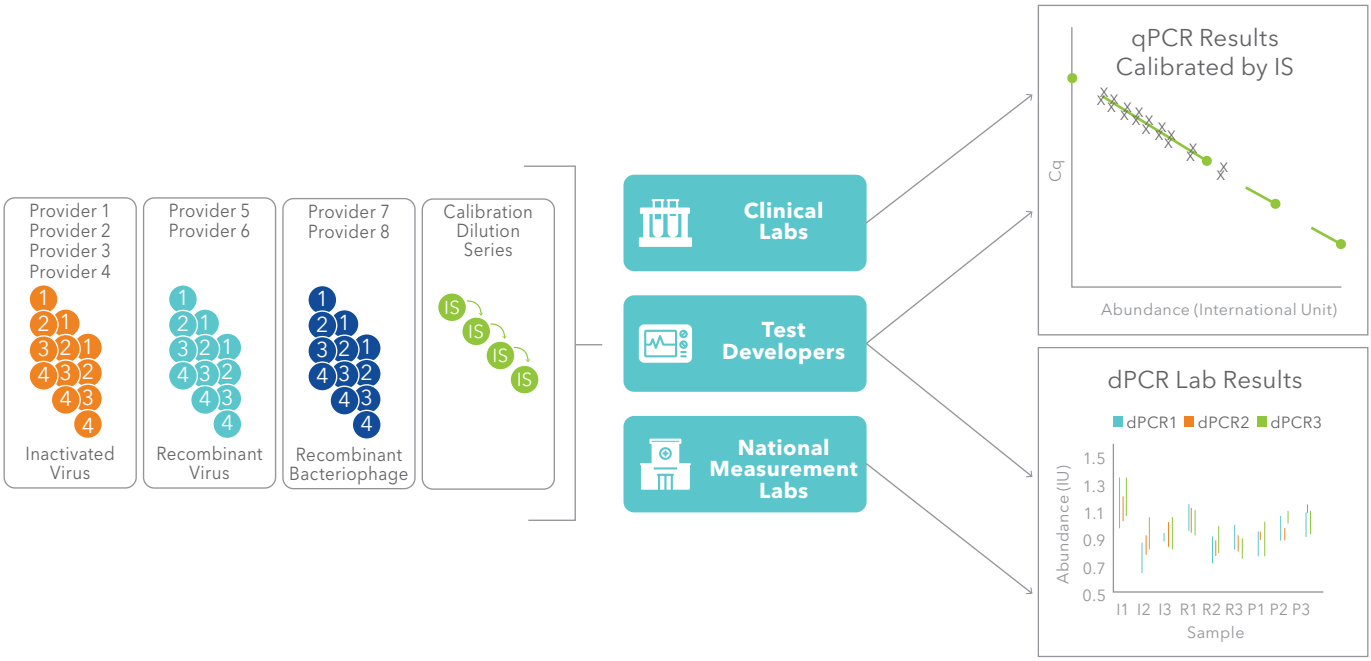


FIGURE 2. Molecular RNA Harmonization Study design outlines sample mix sent and results expected.

Data Processing

Participants were instructed to run all controls in quadruplicate per run. Some laboratories submitted multiple runs' worth of data, while others had some replicate dropout. For the purposes of this analysis, all results were considered even if not all replicates were present. In addition to the variability in testing platform and chemistry, the regions tested across the laboratories varied widely. The most common gene assayed was the nucleocapsid region. For the purpose of further analysis, these results are filtered by nucleocapsid data only.

Data were processed at Stanford prior to dissemination. For digital PCR results, copy numbers derived from droplet quantification were normalized to the WHO-IS standard curve run at each institution and multiplied by dilution factor (if any) used at that institution. The results were log-transformed and reported as observed log10 copies/mL. For real-time PCR results, cycle threshold (Ct) or quantification cycle (Cq) values are reported in log2. These were similarly normalized to the WHO-IS standard curve run at each institution and multiplied by dilution factor used there. The results were log-transformed and reported as observed log10 copies/mL.

Results

Triplicate concentration measurements of the Asuragen Armored RNA Quant SARS-CoV-2 control were reported by each institution and plotted (FIGURE 3). In general, replicates within each institution were very tight. Two notable exceptions were Lab10 and Lab11, where RNA extraction and testing were performed on the same platform. Still, the actual average log concentration for the Asuragen control across all laboratories was 10.2 compared to the expected 10.3 nominal value, with a standard deviation of 0.455 (TABLE 1).

All vendor-submitted control material performed well compared to expected nominal values of the nucleocapsid gene (FIGURE 4, TABLE 1). The slope of the best fit line was 0.97 and the R2 was 0.95.

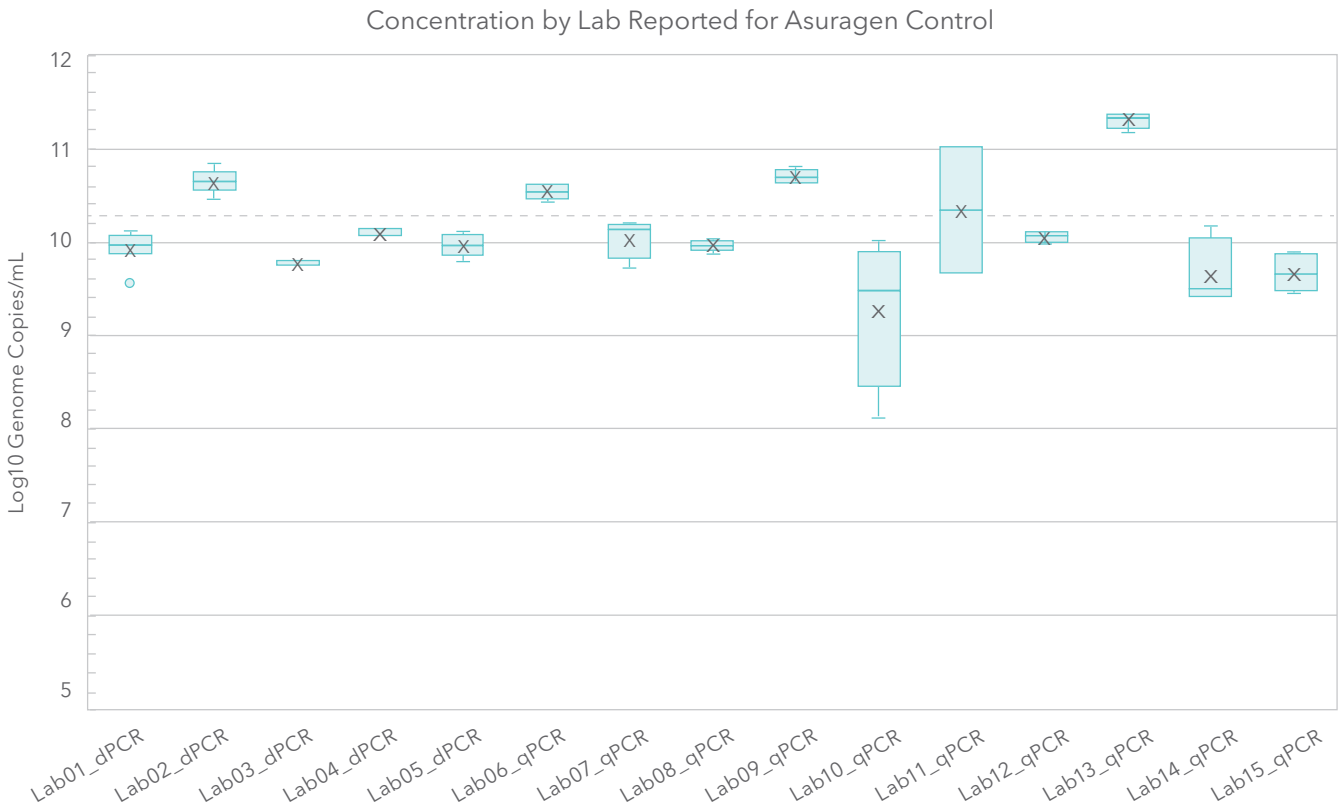


FIGURE 3. Observed Log10 copies/mL as reported across all laboratories. Expected value of 10.3 is represented by the dotted line. Lab07 and Lab08 represent two datasets from the same institution.

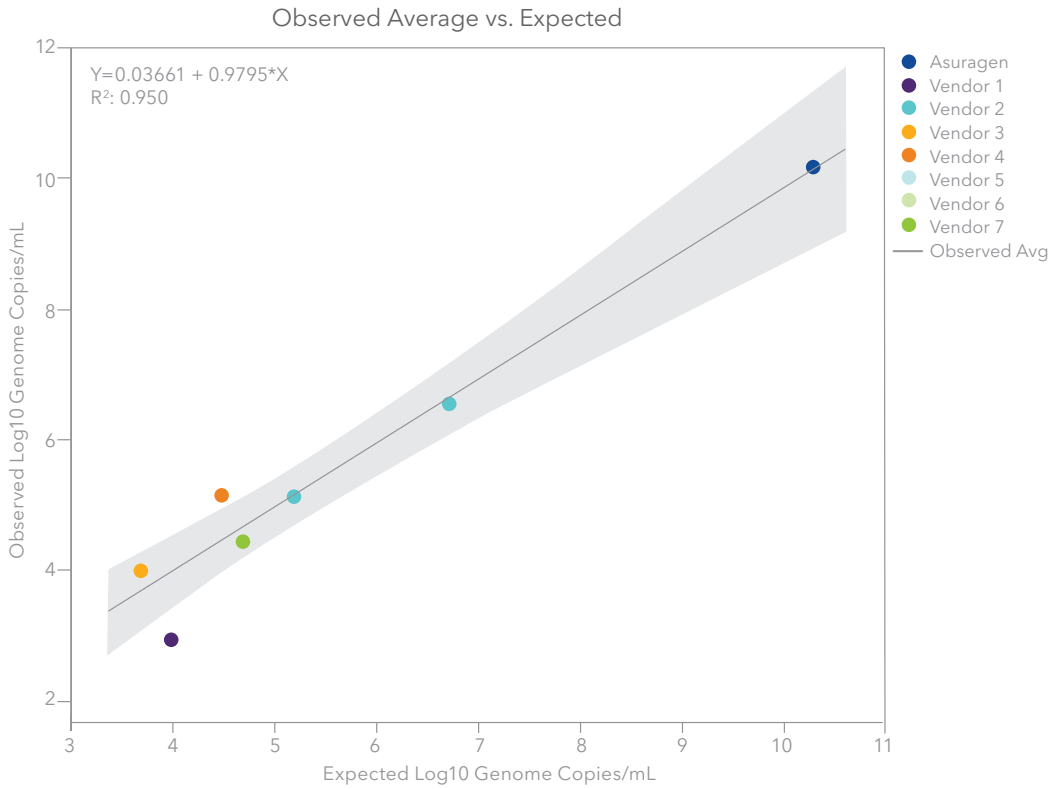


FIGURE 4. Observed vs expected log10 copies/mL across all material vendors.

Vendor	Average Expected Log10 Copies/mL	Average Observed Log10 Copies/mL	Stdev of Observed Log10 Copies/mL	CV
Asuragen	10.3	10.2	0.455	4.5%
Vendor 1	4	2.9	0.710	24.5%
Vendor 2	5.2	5.1	0.267	5.2%
Vendor 3	3.7	4.0	0.249	6.2%
Vendor 4	4.5	5.1	0.441	8.6%
Vendor 5	6.73	6.5	0.748	11.5%
Vendor 6	3.7	4.0	0.337	8.4%
Vendor 7	4.7	4.4	0.355	8.1%

TABLE 1. Observed vs expected log10 copies/mL for each material vendor. Averages are represented from all 15 data sets provided.

Discussion

Despite the differences in quantification methods of starting material, dilution schema, assays and platforms used, detection chemistry involved, and laboratory location, all of the controls submitted to this multisite global study demonstrated good linearity, accuracy, and precision.

The data summary shows that synthetic virus-like particles and bacteriophage encapsulated RNA controls such as the Armored RNA perform just as well in the hands of laboratorians as inactivated virus. Molecular detection using digital and quantitative PCR are extremely sensitive methods, providing data down to 5000 copies/mL. Only one control showed significant difference in log10 copies/mL observed between digital and real-time PCR (data not shown).

The gaps seen in this study seem to reflect differences in testing. A pitfall of using full-length viral controls at the lower end of detection is replicate dropout and increased intra-laboratory variability. A challenge of using synthetic controls is that not all genes represented in every test will necessarily be included in the makeup of the control, causing some tests to result in QC failures.

Ultimately, in order to get a rapid yet accurate diagnosis, it may not be crucial to hit these higher replicative precision metrics since qualitative assays are acceptable to use. What is crucial, though, is that the reference material must be able to serve as full-process analytical controls and consistently be detected over and over in the same range to give confidence in the resulting patient data.

Having a large reserve or near-infinite supply of inactive virus for use as a control may not be possible early in the test development stage. For this reason, synthetic Armored controls were needed at the outset of this pandemic to support test development on a large scale. How these types of controls can be rapidly produced and widely distributed, as well as how to quickly agree on a consensus sequence for broad applicability, will be important considerations in future pandemic response planning.

References

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