The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

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MMed in Virological Pathology

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Abbreviations

CN: Cycle number

Ct: Cycle threshold

E: Envelope

NA: Nucleic acid

NPA: Negative percent agreement

NP: Nasopharyngeal

N: Nucleocapsid

OP: Oropharyngeal

PPA: Positive percent agreement

RdRp: RNA-dependent RNA-polymerase

RP-IC: Internal control

RSP: Rapid sample preparation and inactivation

RT-PCR: Reverse-transcriptase polymerase chain reaction

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

Symbols

™: Trademark

®: Registered trademark

°C: Degrees Celsius

μ: Micro

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Abstract

The SARS-CoV-2 pandemic has resulted in shortages of both critical reagents for nucleic acid purification and highly trained staff as supply chains are strained by high demand, public health measures and frequent quarantining and isolation of staff. This created the need for alternate workflows with limited reliance on specialised reagents, equipment and staff. We present here the validation and implementation of such a workflow for preparing samples for downstream SARS-CoV-2 RT-PCR using liquid handling robots. The rapid sample preparation and inactivation technique evaluated, which included sample centrifugation and heating prior to RT-PCR, showed a 97.37% (95% CI: 92.55-99.28%) positive percent agreement and 97.30% (95% CI: 90.67-99.52%) negative percent agreement compared to nucleic acid purification-based testing. A total of 195 samples were tested as part of the validation. This method was subsequently adopted as the primary sample preparation method in the Groote Schuur Hospital Virology Diagnostic Laboratory in Cape Town, South Africa.

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), an emergent betacoronavirus, was identified as a novel causative agent of severe pneumonia in Wuhan, China in 2019 [1]. The capacity for person-to-person transmission was soon identified and the ensuing pandemic has caused more than seventeen million cases at the time of submission [2].

Currently, diagnostic testing for SARS-CoV-2 relies on molecular techniques, primarily reverse-transcriptase polymerase chain reaction (RT-PCR), from respiratory specimens [3]. The specialised equipment and reagents required to offer these tests at scale has placed significant strain on worldwide supply chains of reagents. Public health measures put in place in numerous countries, including travel restrictions, have further made planning for sustainable service delivery difficult as laboratory stock orders may not be filled on time. These issues motivate for the use of diagnostic workflows that favour locally or readily available reagents to, at least partially, insulate supply chains from fluctuations in global demand and evolving travel limiting public health measures. To address these issues, a number of laboratories have successfully developed alternative sample preparation techniques which limit reagent needs and avoid complex nucleic acid (NA) purification protocols [4-6]. There is also a significant cost saving when the reagent-free direct heating method, as described by Fomsgaard and Rosenstierne [4], is used which will become critical if economic fallout from the pandemic intensifies. Staff shortages in the laboratory are an inevitability as social distancing requirements are implemented in concert with increasing demand for diagnostic testing. SARS-CoV-2 outbreaks in the laboratory environment may also introduce unpredictable shortages of critical

staff further limiting the capacity of laboratories to offer predictable test turnaround times. The necessary influx of new staff, who may have limited training or training in a related field, can further compromise the reliability of diagnostic laboratory services as the capacity for oversight and quality control is hindered by rapidly evolving testing demands and workflow instability due to reagent shortages and potentially unreliable testing kits due to limited regulatory oversight [7]. All these factors highlight the need for automated workflows that limit the number of laboratory staff-dependent steps and in particular steps requiring specialised training. Automation further limits human error such as sample switches and cross-contamination and are generally amenable to greater degrees of workflow control due to traceable instrument log files.

A chemical reagent-free heat-based rapid sample preparation and inactivation (RSP) [8, 9] method for downstream SARS-CoV-2 RT-PCR amplification is presented here optimised for use on automated liquid handling robots.

Materials and methods

Ethics

Biological material of human origin was anonymised and all clinical and other personally identifiable data delinked with only study specific sample identifiers used along with sample SARS-CoV-2 assay performance data. Ethics approval for this work was granted by the University of Cape Town Human Research Ethics Committee (HREC reference number: 335/2020).

Sample selection

Nasopharyngeal (NP) and oropharyngeal (OP) swabs sent dry or in saline to the National Health Laboratory Service Virology Diagnostic Laboratory in Groote Schuur Hospital from its standard referral area for SARS-CoV-2 testing were included. Selection of 115 samples, which tested positive, and 80 samples, which tested negative, for SARS-CoV-2 by NA purification-based commercial diagnostic assays in use at the diagnostic laboratory was done for the method validation. Spectrum bias was avoided by selecting consecutive samples that tested positive by standard testing over two discrete intervals of regular laboratory workflow. Samples that tested negative were selected randomly from the same intervals. The diagnostic assays in use were the Abbott RealTime SARS-CoV-2 Assay (Abbott Laboratories, USA) running on the Abbott m2000 RealTime system and the Allplex™ 2019-nCoV assay (Seegene, South Korea). The assays were run as per package insert (Quick Manual

Version 1.0). The Allplex™ 2019-nCoV assay was performed after sample NA purification using the NucliSENS® easyMag® (bioMérieux, France) as per package insert.

Rapid sample preparation and inactivation

Standard diagnostic testing sample preparation included placing NP or OP swabs in a 2ml Sarstedt sample tube containing 1.5ml autoclaved 0.9% saline. If both a NP and OP swab or multiple swabs of the same type was received, they were combined in a single tube. The swabs were cut to fit in the tube. The tube was then vortexed for 10 seconds. The saline was used as the sample input for downstream assays after which the tube was stored at 4°C. Stored tubes from diagnostic samples were available for inclusion in the study.

Selected sample tubes were centrifuged at 16 000 *g* for 5 minutes and 50µl of the supernatant was then pipetted into the wells of a 96-well PCR plate. The PCR wells were capped and the plate incubated on a thermocycler at 98°C for 5 minutes followed by 4°C for 2 minutes. The PCR plate was then briefly centrifuged and placed on a dedicated QIAgility (Qiagen, Germany) liquid handling instrument for sample-addition.

RT-PCR after rapid sample preparation and inactivation

Concurrent with sample preparation, a second dedicated QIAgility instrument was used for Allplex™ 2019-nCoV assay master mix preparation and aliquoting into appropriate 8-well PCR strips (Bio-Rad Laboratories, USA). Following master mix preparation, the PCR strips were transferred to the sample-addition QIAgility instrument. The sample input volume and master mix constituents are shown in Table 1.

Table 1. RT-PCR reaction preparation

	Volume per reaction (µl)
RNase-free Water	11.1
2019-nCoV MOM (primer and probe mix)	6
5X Real-time One-step Buffer	6
Real-time One-step Enzyme	2.4
Internal control (RP-IC)	1.5
Sample after centrifugation and heating	3
Total volume	30

After sample addition, the PCR strips were sealed and briefly centrifuged before being loaded on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The real-time PCR cycling parameters recommended by the Allplex™ 2019-nCoV assay package insert (Quick Manual Version 1.0) were used unchanged. Real-time data analysis was performed using the 2019-nCoV Viewer for

Real time Instruments V3 (Ver 3.18.005.003) software as per the Allplex™ 2019-nCoV assay package insert (Quick Manual Version 1.0).

If the internal control (RP-IC) was not detected with a cycle threshold (Ct) value <40 and no SARS-CoV-2 targets were detected, the test was deemed invalid and the primary sample was retested with a decreased sample volume input, 2μl instead of 3μl, to reduce the concentration of PCR inhibitors in the reaction. The remainder of the protocol was unchanged.

Repeatability and analytical sensitivity

Inter-assay reproducibility was assessed using 8 samples with Envelope (E) gene Ct values ranging between 17.16 and 35.63, which were tested in triplicate 7 days after initial testing. Intra-assay reproducibility was assessed by repeating 16 samples in triplicate. Samples were stored at 4°C while awaiting repeat testing. To assess relative analytical sensitivity, one sample with a mean Ct value of 33.9 for the assay targets was selected and serially diluted with saline and tested with multiple replicates at dilutions specifically selected to allow calculation of the analytical sensitivity of the Allplex™ 2019-nCoV assay after NA purification and RSP. The dilution at which SARS-CoV-2 RNA could be detected with 95% confidence was determined for each method by Probit analysis. The absolute analytical sensitivity of the RSP method was then calculated based on the relative analytical sensitivity compared to NA purification-based detection. The absolute analytical sensitivity for NA purification-based detection is reported in the Allplex™ 2019-nCoV assay package insert (Quick Manual Version 1.0).

Statistical analysis and graphics

Data visualisation and statistical analysis, including paired t-tests for comparison of target Ct values, a Fisher's exact test for statistical significance determination of the positive percent agreement (PPA) and negative percent agreement (NPA) with NA extraction-based testing and the Wilson/Brown method for 95% confidence interval determination, was done using GraphPad Prism version 8.4.2 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com. Probit analysis was performed using R version 4.0.2 in R Studio [10].

Results and discussion

The RSP method validation included 115 serially collected samples which tested positive and 80 randomly selected samples from the same period which tested negative for SARS-CoV-2 by NA purification-based testing. After testing with the RSP method, repeat testing with a decreased sample volume was required for 20 of the 195 (10.26%) samples due to detection of neither SARS-CoV-2 targets nor the internal control. One sample could not be tested using the RSP method due to excessive viscosity from nasopharyngeal swab breakdown. Repeat testing failed to generate a result for 6 samples possibly due to sample-specific PCR inhibition. The Allplex™ 2019-nCoV assay result after RSP correlated with that of NA purification-based testing for 111 positive and 72 negative samples as shown in Table 2. No result could be generated for 7 of 195 (3.59%) samples. Raw data is shown in the S1 Appendix.

Table 2. Contingency table used for positive and negative percent agreement with NA purification-based testing calculation

	Positive SARS-CoV-2 Abbott RealTime SARS-CoV-2 Assay or Seegene Allplex™ 2019-nCoV Assay NA Purification	**Negative SARS-CoV-2 Abbott RealTime SARS-CoV-2 Assay or Seegene Allplex™ 2019-nCoV Assay NA Purification**
Positive SARS-CoV-2, RSP method, Seegene Allplex™ 2019-nCoV Assay	111	2
Negative SARS-CoV-2, RSP method, Seegene Allplex™ 2019-nCoV Assay	3	72

The PPA and NPA of the RSP method with NA purification-based testing for SARS-CoV-2 demonstrated a P value of <0.0001. The PPA of the RSP method was 97.37% (95% CI: 92.55-99.28%) and the NPA 97.30% (95% CI: 90.67-99.52%). The 7 samples, for which no result could be generated by RSP due to repeated invalid results or sample unsuitability, were excluded from this analysis as standard laboratory practice designates samples for NA purification-based testing in cases of RSP failure.

The Ct values of individual targets of the Allplex™ 2019-nCoV assay were assessed for samples prepared by NucliSENS® easyMag® NA purification and RSP. The E gene, RNA-dependent RNA-polymerase (RdRp) gene and Nucleocapsid (N) gene targets had Ct values that were significantly different with a P value of <0.0001 (Fig 1). The mean difference in Ct values between RSP and NA purification was 2.148 (95% CI: 1.909-2.387) for the E gene, 3.271 (95% CI: 3.037-3.506) for the RdRp gene and 1.608 (95% CI: 1.407-1.809) for the N gene, with RSP demonstrating a higher mean Ct value in each case.

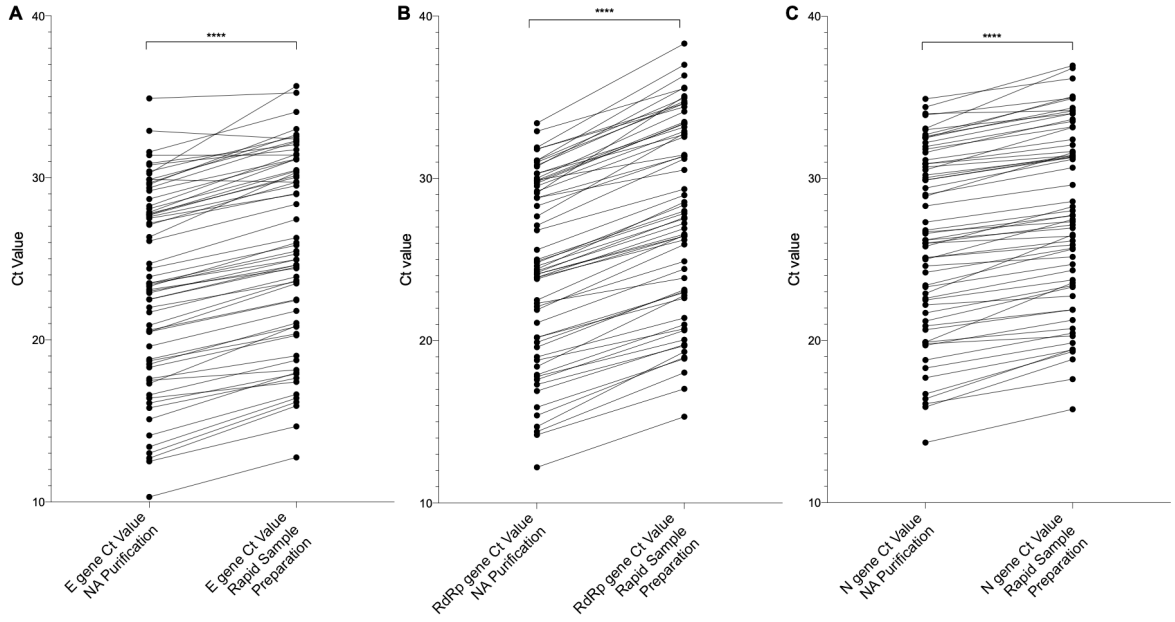

Fig 1. Comparison of target Ct values after RSP and NucliSENS® easyMag NA purification. The Ct values for the SARS-CoV-2 (A) Envelope (E), (B) RNA-dependent RNA-polymerase (RdRp) and (C) Nucleocapsid (N) gene targets are shown for samples tested with the Allplex™ 2019-nCoV assay after NucliSENS® easyMag® NA purification and RSP. The difference in generated Ct values was found to be statistically significant in each case with a P value of <0.0001 as determined by paired t-test.

The relative performance of the Abbott RealTime SARS-CoV-2 assay and the Allplex™ 2019-nCoV assay after RSP is shown in Fig 2. The Abbott assay reports cycle number (CN) values which are not equivalent to Ct values and thus are not directly comparable.

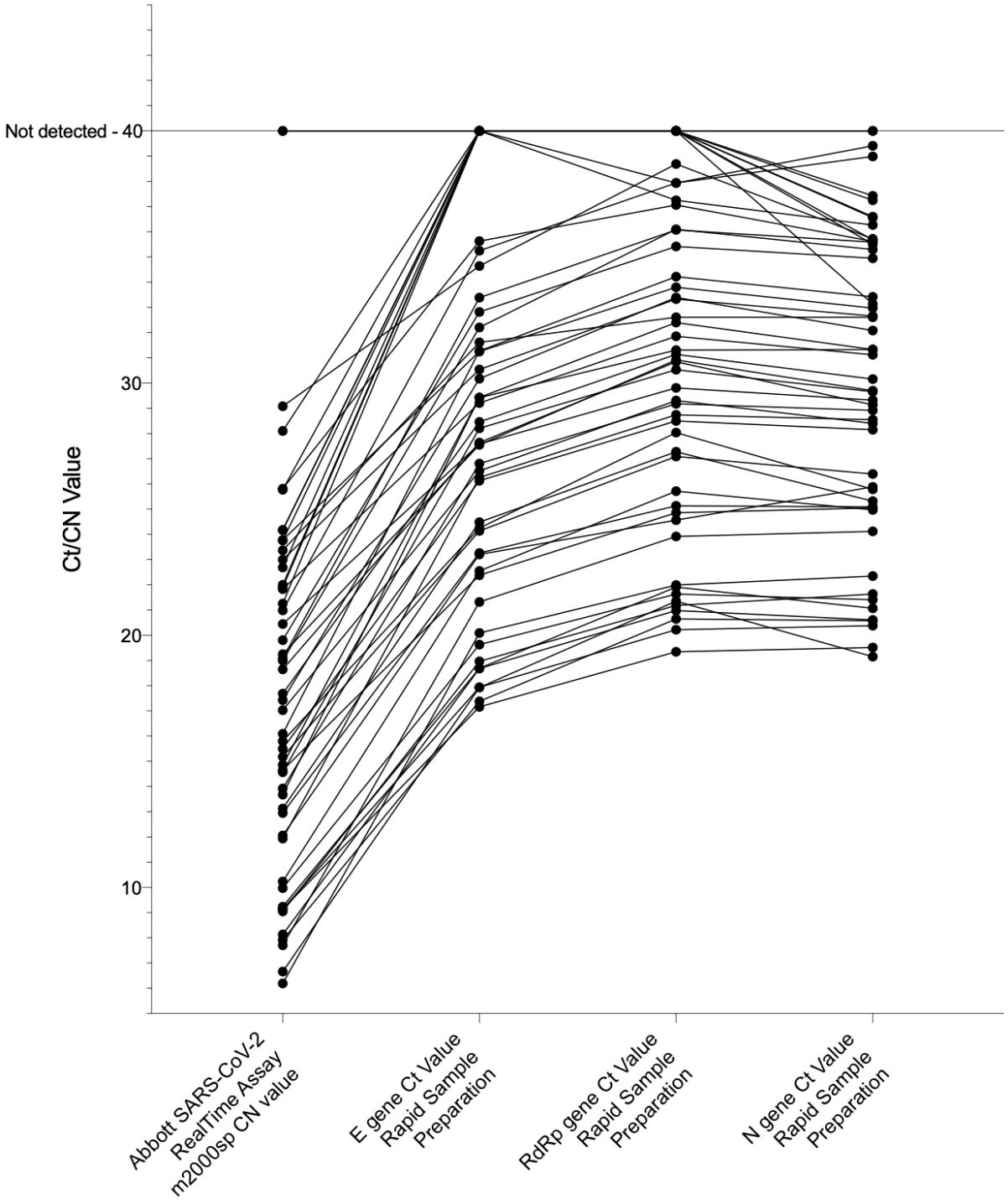

Fig 2. Comparison of target Ct and CN values after RSP and testing with the Abbott RealTime SARS-CoV-2 assay. The Ct values for the SARS-CoV-2 Envelope (E), RNA-dependent RNA-polymerase (RdRp) and Nucleocapsid (N) gene targets are shown for samples tested with the Allplex™ 2019-nCoV assay after RSP and CN values after testing with the Abbott RealTime SARS-CoV-2 assay. A plotted CN or Ct value of 40 indicates that detectable amplification did not occur. The Abbott assay CN values are assay specific and not directly comparable to Ct values, but are shown to demonstrate the performance of the spectrum of selected samples.

The single false negative result from the RSP method when compared to NucliSENS® easyMag® NA purification was from a sample that only tested positive for one of the three Allplex™ 2019-nCoV targets, the N gene, with a Ct value of 36.7. The two false negatives from the RSP method when compared to the Abbott RealTime SARS-CoV-2 Assay, which includes NA purification, had high CN values. However, samples with higher CN values were detected thus sample-specific inhibition may also have played a role.

There were two false positive results from the RSP method when compared to the Abbott RealTime SARS-CoV-2 Assay. A single target was detected in both cases with Ct values above 35. This may represent contamination events or the samples may have viral RNA at levels near the limit of detection for both assays. NA contamination in the laboratory is monitored for by frequent testing of environmental swabs and reagent blanks. Multiple negative controls are also included in each run.

The intra-assay repeatability assessment of mean Ct values for the three Allplex™ 2019-nCoV targets showed a coefficient of variance of 1.14%. The inter-assay repeatability assessment of mean Ct values after 7 days of sample storage showed a coefficient of variance of 1.27%.

The relative analytical sensitivity of the Allplex™ 2019-nCoV assay after RSP was found to be 583 RNA copies per reaction. This was calculated from the 5.83-fold decrease in analytical sensitivity of the RSP method compared to NucliSENS® easyMag® NA purification-based testing, which has an analytical sensitivity of 100 RNA copies per reaction as per the Allplex™ 2019-nCoV assay package insert (Quick Manual Version 1.0). The relative decrease was determined by serially diluting and testing a sample with multiple replicates as shown in Table 3. This relative loss in analytical sensitivity can largely be explained by the smaller sample input volume for RSP. NucliSENS® easyMag® NA purification concentrates sample nucleic acids by a factor of approximately 2, based on sample input versus elution volume. Additionally, the Allplex™ 2019-nCoV assay input volume after NA purification is 8μl versus the 3μl sample input volume for RSP. Thus, the expected loss in analytical sensitivity would be 5.3-fold which is comparable to the experimentally determined loss of 5.83-fold and suggests that sample inhibition plays a minor role. Raw data is shown in the S2 Appendix.

Table 3. Relative analytical sensitivity assessment

Dilution	Replicates	Seegene Allplex™ 2019-nCoV Assay RSP Method Percentage of Samples Positive	Seegene Allplex™ 2019-nCoV Assay NA Purification Percentage of Samples Positive
1:20	24	100%	Not done
1:40	24	95.8%	Not done
1:80	24	70.8%	Not done
1:120	24	58.3%	Not done
1:160	24	41.7%	Not done
1:200	10	Not done	100%
1:320	24	33.3%	Not done
1:400	10	Not done	100%
1:500	10	Not done	90%
1:625	10	Not done	70%
1:2000	10	Not done	60%
1:5000	10	Not done	30%

The performance characteristics were deemed acceptable for clinical diagnostic use in the Groote Schuur Hospital Virology Diagnostic Laboratory and allowed the laboratory to increase the number of samples tested daily by a factor of 5-10 due to the decreased supply chain dependence and simplified workflow. While large quantities of some consumables were still required, such as liquid handling robot tips for the QIAgility instruments, the availability of generic alternatives and the fact that

they are neither SARS-CoV-2 specific nor universally required made consumable depletion less of a concern. The reduced processing time further facilitated a more rapid test turnaround time which was beneficial for in-hospital infection control. A stable workflow, not subject to reagent availability dependent variations, also decreased laboratory errors and may allow for improved clinical planning as a result of a stable test turnaround time.

Prior to the automation described in this protocol, earlier versions of the RSP method were susceptible to fluctuating failure rates. This was largely due to human errors arising from staff shortages and rising test volumes. A simple automated workflow was needed to enable staff with minimal molecular experience to be able to perform testing reliably. In particular the time intervals between assay steps and how thoroughly the master mix was mixed prior to aliquoting were identified as sources of assay performance variation. This operator dependency and fluctuating staff availability motivated for the further automation of the process with liquid handling robots and ultimately the validation described here.

The laboratory approach to result interpretation was also affected by the implementation of the RSP method. The approach to NucliSENS® easyMag® NA purification-prepared samples involved release of numerous inconclusive results, despite multiple target amplification at times, due to the known capacity for sample contamination both on the easyMag® instrument and during processing of swabs. The known decrease in sensitivity of the RSP method and the lack of use of the easyMAG® open system for processing, decreased the number of low-level contamination induced inconclusive results released by our laboratory.

The limitations of this study include evaluation of only a single commercial kit at a single site. Future research should thus investigate alternative RT-PCR commercial kits, in-house assays and equipment. Limitations of the RSP method include unsuitability for sample types other than swabs, such as sputum, and lack of automation of initial processing steps including the placing of dry swabs in labelled saline containers. Further, a poorer analytical sensitivity may be relevant in certain clinical situations such as late presentations.

NA purification is the gold-standard in sample processing for RT-PCR, however, in the setting of a pandemic with significant pressures on reagent supply chains and the need for a rapid increase in testing capacity, the RSP method described here presented a reasonable alternative and has been implemented as the primary sample preparation method in the Groote Schuur Hospital Virology Diagnostic Laboratory in South Africa.

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Supporting information

S1 Appendix. Sample cycle threshold and cycle number values for SARS-CoV-2 targets and internal controls. The cycle threshold (Ct) and cycle number (CN) values of assay targets and internal controls from the Allplex™ 2019-nCoV and Abbott RealTime SARS-CoV-2 assays respectively are shown for samples used. The mastermix protocol used is also shown. RSP: Rapid sample preparation and inactivation.

S2 Appendix. Sample cycle threshold values at dilutions used for analytical sensitivity determination. The cycle threshold (Ct) values for the Allplex™ 2019-nCoV assay targets and internal control at dilutions used in the determination of the analytical sensitivity of the rapid sample preparation and inactivation (RSP) method relative to nucleic acid purification.

Appendix A

Supplementary tables

	Seegene Allplex 2019-nCoV assay with RSP Protocol 1 - primary protocol				Seegene Allplex 2019-nCoV assay with RSP Protocol 2 - invalid repeat testing				Seegene Allplex 2019-nCoV assay NucliSENS® easyMag® nucleic acid purification				Abbott RealTime SARS-CoV-2 Assay	
	E gene Ct value	RdRp gene Ct value	N gene Ct value	Internal control Ct value	E gene Ct Value	RdRp gene Ct Value	N gene Ct Value	Internal control Ct value	E gene Ct value	RdRp gene Ct value	N gene Ct value	Internal control Ct Value	CN value	Internal control Ct Value
Sample 1	34.07	Not detected	34	31.68	not done	not done	not done	not done	31.6	32.1	33	28.1	not done	not done
Sample 2	19.02	21.4	21.9	Not detected	not done	not done	not done	not done	17.6	19	20.66	Not detected	not done	not done
Sample 3	23.66	26.5	25.64	Not detected	not done	not done	not done	not done	20.9	22.5	23.4	34	not done	not done
Sample 4	29.74	31.45	31.18	28.27	not done	not done	not done	not done	27.1	29.9	29.4	28.8	not done	not done
Sample 5	28.38	31.2	30.66	36.14	not done	not done	not done	not done	26.1	27.1	29	27.1	not done	not done
Sample 6	21.04	23.03	23.3	31.21	not done	not done	not done	not done	18.5	19.9	21.2	Not detected	not done	not done
Sample 7	16.16	19.32	19.45	Not detected	not done	not done	not done	not done	13	14.7	16.4	Not detected	not done	not done
Sample 8	12.74	15.32	15.76	Not detected	not done	not done	not done	not done	10.3	12.2	13.7	Not detected	not done	not done
Sample 9	16.4	18.97	19.32	Not detected	not done	not done	not done	not done	13.4	15.4	16.7	Not detected	not done	not done
Sample 10	30.04	35.06	32.06	37.07	not done	not done	not done	not done	27.8	29.1	30.9	34.6	not done	not done
Sample 11	21.79	24.42	24.33	33.21	not done	not done	not done	not done	19.6	21.1	22.6	29.4	not done	not done
Sample 12	14.66	17.03	17.62	Not detected	not done	not done	not done	not done	12.5	14.2	16.1	Not detected	not done	not done
Sample 13	26	28.54	28.26	26.34	not done	not done	not done	not done	23.3	24.4	25.8	34.5	not done	not done
Sample 14	31.41	34.75	34.16	26.46	not done	not done	not done	not done	31.4	30.8	32.6	27.5	not done	not done
Sample 15	28.99	30.52	31.35	25.27	not done	not done	not done	not done	27.5	28.8	29.9	33.3	not done	not done
Sample 16	24.42	27.23	27.13	24.93	not done	not done	not done	not done	23.1	24.3	26	25.2	not done	not done
Sample 17	25.84	27.99	27.7	25.97	not done	not done	not done	not done	23.9	25	26.6	37.4	not done	not done
Sample 18	17.98	19.76	20.46	Not detected	not done	not done	not done	not done	15.1	16.9	18.8	Not detected	not done	not done
Sample 19	32.05	34.61	34.18	24.68	not done	not done	not done	not done	30.9	31.8	34	28.3	not done	not done
Sample 20	20.37	22.62	22.74	23.53	not done	not done	not done	not done	18.7	20.2	22.2	Not detected	not done	not done
Sample 21	24.61	26.56	26.44	24.76	not done	not done	not done	not done	23	24.1	26	32.7	not done	not done
Sample 22	17.4	20.06	20.28	30.71	not done	not done	not done	not done	16.4	17.8	19.8	Not detected	not done	not done
Sample 23	24.5	26.21	26.15	24.62	not done	not done	not done	not done	22.5	23.9	25.1	33.4	not done	not done
Sample 24	31.16	32.69	33.18	25.01	not done	not done	not done	not done	29.2	30.3	31.8	29.5	not done	not done
Sample 25	20.28	23.14	23.73	25.43	not done	not done	not done	not done	18.3	19.6	22.5	Not detected	not done	not done
Sample 26	22.49	24.89	25.16	24.3	not done	not done	not done	not done	20.6	22.1	24.6	35.8	not done	not done
Sample 27	18.14	20.73	21.9	26.91	not done	not done	not done	not done	17.5	18.8	20.9	39	not done	not done
Sample 28	29.04	31.39	31.3	26.25	not done	not done	not done	not done	27.2	28.3	30.2	29.3	not done	not done
Sample 29	31.74	Not detected	35.05	31.08	not done	not done	not done	not done	30.8	31.7	32.5	29.2	not done	not done
Sample 30	20.81	22.97	23.39	23.52	not done	not done	not done	not done	18.8	20.2	21.7	38.3	not done	not done
Sample 31	25.3	27.52	27.73	25.03	not done	not done	not done	not done	23.4	24.6	26.2	26.2	not done	not done
Sample 32	22.44	23.86	24.7	24.37	not done	not done	not done	not done	20.5	22.3	23.3	32.3	not done	not done
Sample 33	32.39	35.54	36.16	26.38	not done	not done	not done	not done	32.9	32.9	34.9	27.7	not done	not done
Sample 34	23.48	25.93	26.53	25.25	not done	not done	not done	not done	20.5	21.9	22.9	33.2	not done	not done
Sample 35	26.3	28.97	28.57	26.93	not done	not done	not done	not done	24.4	25.6	27.3	28	not done	not done
Sample 36	16.63	18.89	19.42	36.22	not done	not done	not done	not done	14.1	15.9	17.7	Not detected	not done	not done
Sample 37	24.95	26.91	27.46	25.52	not done	not done	not done	not done	22.9	23.9	25	31.3	not done	not done
Sample 38	23.9	26.45	25.69	25.18	not done	not done	not done	not done	21.7	23.8	24.2	30.6	not done	not done
Sample 39	32.29	34.95	34.35	26.28	not done	not done	not done	not done	29.9	31.1	32.5	27.8	not done	not done
Sample 40	15.92	18.03	18.84	Not detected	not done	not done	not done	not done	12.7	14.4	15.9	Not detected	not done	not done
Sample 41	29.52	31.35	31.29	25.42	not done	not done	not done	not done	27.6	28.8	29.9	27.5	not done	not done
Sample 42	18.74	20.99	21.26	30.62	not done	not done	not done	not done	16.6	17.9	19.7	Not detected	not done	not done
Sample 43	17.64	19.69	19.85	32.41	not done	not done	not done	not done	15.8	17.3	18.3	Not detected	not done	not done
Sample 44	33.01	34.61	34	25.77	not done	not done	not done	not done	29.6	31	32.2	28.9	not done	not done
Sample 45	35.25	38.3	36.96	29.36	not done	not done	not done	not done	34.9	33.4	34.4	28.7	not done	not done
Sample 46	20.86	22.79	23.56	23.89	not done	not done	not done	not done	17.3	18.4	19.9	Not detected	not done	not done
Sample 47	27.44	29.34	29.6	28.96	not done	not done	not done	not done	24.7	26.8	28.3	27.9	not done	not done
Sample 48	25.48	28.36	28.01	27.71	not done	not done	not done	not done	23.5	24.8	26.8	31.2	not done	not done
Sample 49	17.9	20.64	20.73	Not detected	not done	not done	not done	not done	16.1	17.6	19.9	Not detected	not done	not done
Sample 50	23.58	26.44	25.9	26.8	not done	not done	not done	not done	22	24.1	25.1	35.3	not done	not done
Sample 51	30.48	32.56	31.66	28.6	not done	not done	not done	not done	28.1	29.2	30.9	28.3	not done	not done
Sample 52	32.56	34.41	34.96	25.02	not done	not done	not done	not done	30.4	31.8	33.9	25.8	not done	not done
Sample 53	24.62	27.59	26.94	27.83	not done	not done	not done	not done	22.5	24.2	26.2	24.9	not done	not done
Sample 54	24.95	27.84	27.33	26.08	not done	not done	not done	not done	23.5	24.9	26.7	36.3	not done	not done
Sample 55	30.15	33.36	31.41	Not detected	not done	not done	not done	not done	27.7	29.8	30.7	Not detected	not done	not done
Sample 56	Not detected	Not detected	Not detected	29.35	not done	not done	not done	not done	Not detected	Not detected	36.7	Not detected	not done	not done
Sample 57	22.55	25.72	24.97	31.1	not done	not done	not done	not done	not done	not done	not done	not done	10.23	20.62
Sample 58	32.21	36.1	35.3	28.93	not done	not done	not done	not done	not done	not done	not done	not done	19	16.99
Sample 59	29.43	32.4	31.33	29.17	not done	not done	not done	not done	not done	not done	not done	not done	13.68	17.54
Sample 60	Not detected	Not detected	36.56	29.65	not done	not done	not done	not done	not done	not done	not done	not done	21.26	16.97
Sample 61	21.32	23.92	24.12	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	7.71	19.38
Sample 62	28.21	30.53	29.66	28.95	not done	not done	not done	not done	not done	not done	not done	not done	14.57	17.5
Sample 63	32.82	35.42	34.95	29.99	not done	not done	not done	not done	not done	not done	not done	not done	19.81	16.73
Sample 64	31.28	34.22	33.42	28.95	not done	not done	not done	not done	not done	not done	not done	not done	16.09	17.07
Sample 65	20.09	21.99	22.35	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	6.19	24.33
Sample 66	29.42	31.31	31.33	30.24	not done	not done	not done	not done	not done	not done	not done	not done	14.86	17.89
Sample 67	Not detected	Not detected	37.25	31.11	not done	not done	not done	not done	not done	not done	not done	not done	24.17	17.14
Sample 68	33.39	36.08	35.59	29.16	not done	not done	not done	not done	not done	not done	not done	not done	19.08	16.7
Sample 69	Not detected	37.94	38.99	29.36	not done	not done	not done	not done	not done	not done	not done	not done	24.18	16.9
Sample 70	35.25	37.94	39.41	29.53	not done	not done	not done	not done	not done	not done	not done	not done	20.99	17.09
Sample 71	26.26	28.74	28.55	27.89	not done	not done	not done	not done	not done	not done	not done	not done	11.94	Not detected
Sample 72	29.73	33.16	33.35	26.82	not done	not done	not done	not done	29.9	30.3	32.6	30.3	not done	not done
Sample 73	27.64	30.85	29.14	Not detected	26.63	29.09	28.68	27.76	not done	not done	not done	not done	18.65	17.89
Sample 73 (inter-run repeat 1)	26.14	29.26	28.24	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 73 (inter-run repeat 2)	26.06	29.13	28.12	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 73 (inter-run repeat 3)	26.23	29.36	28.24	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 74	17.38	20.65	20.58	Not detected	17.09	19.96	20.36	Not detected	not done	not done	not done	not done	7.92	21.36
Sample 74 (inter-run repeat 1)	16.57	19.95	20.03	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 74 (inter-run repeat 2)	17.74	20.29	20.21	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 74 (inter-run repeat 3)	16.95	19.94	20.01	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 75	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	31.57	not done	not done	not done	not done	22.69	17.36
Sample 76	35.63	37.06	35.62	32.17	33.17	34.93	34.12	28.03	not done	not done	not done	not done	25.81	17.31
Sample 76 (inter-run repeat 1)	33.7	36	35.88	32.97	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 76 (inter-run repeat 2)	34.84	37.05	34.71	33.82	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 76 (inter-run repeat 3)	35.09	36.76	36.53	35.77	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 77	Not detected	Not detected	37.43	Not detected	Not detected	Not detected	36.68	28.49	not done	not done	not done	not done	Not detected	17.12
Sample 78	Not detected	Not detected	36.6	Not detected	Not detected	36.73	34.73	29.85	not done	not done	not done	not done	23.76	17.12
Sample 79	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	35.68	30.38	not done	not done	not done	not done	Not detected	17.43
Sample 80	17.16	19.35	19.52	Not detected	17.04	19.12	20.03	Not detected	not done	not done	not done	not done	9.14	20.16
Sample 80 (inter-run repeat 1)	17.45	19.26	19.65	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 80 (inter-run repeat 2)	16.91	19.31	19.42	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 80 (inter-run repeat 3)	17.97	19.61	19.66	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 81	Invalid	Invalid	Invalid	Not detected	Not detected	37.25	36.27	28.25	not done	not done	not done	not done	21.9	17.45
Sample 82	24.25	28.04	25.79	Not detected	24.2	27.03	25.99	28.28	not done	not done	not done	not done	15.8	17.85
Sample 82 (inter-run repeat 1)	24.36	27.75	25.51	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 82 (inter-run repeat 2)	24.26	27.69	25.49	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 82 (inter-run repeat 3)	25.04	27.95	25.76	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 83	24.49	27.28	25.32	Not detected	23.77	26.12	25.24	25.85	not done	not done	not done	not done	13.13	19.16
Sample 84	Not detected	Not detected	35.51	Not detected	32.4	35.23	34.38	30.32	not done	not done	not done	not done	28.1	17.11
Sample 85	18.97	21.18	21.64	Not detected	18.02	20.45	21.42	34.68	not done	not done	not done	not done	9.05	19.73
Sample 86	27.59	29.81	29.33	27.63	27.53	29.17	29.15	26.45	not done	not done	not done	not done	20.45	18.11
Sample 87	22.38	24.86	25.05	Not detected	22.21	23.64	24.65	30.13	not done	not done	not done	not done	14.65	18.58
Sample 88	27.55	30.92	29.7	30.42	26.97	29.97	29.54	27.28	not done	not done	not done	not done	19.25	17.79
Sample 89	26.52	29.31	28.4	Not detected	26.12	28.12	28.24	28.04	not done	not done	not done	not done	17.03	17.32
Sample 90	Not detected	Not detected	33.14	Not detected	31.3	Not detected	32.29	Not detected	not done	not done	not done	not done	22	17.32
Sample 91	18.69	21.91	21.07	Not detected	18.71	20.81	20.54	Not detected	not done	not done	not done	not done	9.24	21.59
Sample 92	30.54	33.33	32.66	Not detected	30.27	33.53	32.52	35.61	not done	not done	not done	not done	23	17.54
Sample 93	18.68	20.98	20.61	Not detected	18.7	21.04	20.53	Not detected	not done	not done	not done	not done	8.14	20.48
Excluded 1	not done too viscous	not done too viscous	not done too viscous	not done too viscous	not done too viscous	not done too viscous	not done too viscous	not done too viscous	not done	not done	not done	not done	24.56	17.25
Sample 94	31.62	32.61	32.61	29.47	30.91	32.33	32.79	29.6	not done	not done	not done	not done	23.37	17.81
Sample 94 (inter-run repeat 1)	29.96	32.41	32.28	29.53	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 94 (inter-run repeat 2)	30.26	32.91	32.63	29.77	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 94 (inter-run repeat 3)	29.83	32.56	32.26	29.41	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 95	29.21	31.86	31.12	31.38	28.81	31.58	30.9	33.42	not done	not done	not done	not done	21.83	17.23
Sample 95 (inter-run repeat 1)	28.85	31.21	30.64	31.17	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 95 (inter-run repeat 2)	28.7	30.94	30.79	30.51	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 95 (inter-run repeat 3)	28.94	31.41	30.83	30.95	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done
Sample 96	26.81	29.18	28.92	28.92	27.76	29.48	29.44	29.68	not done	not done	not done	not done	13.92	20.32
Sample 97	26.13	28.5	28.15	28.15	25.98	28.61	28.27	28.64	not done	not done	not done	not done	15.5	18.2
Sample 98	24.14	27.09	26.4	26.4	23.11	26.11	25.5	31.91	not done	not done	not done	not done	15.18	20.13
Sample 99	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	30.81	not done	not done	not done	not done	25.77	17.72
Sample 100	17.93	21.35	19.15	19.15	17.17	19.93	18.74	Not detected	not done	not done	not done	not done	6.66	21.08

RSP Protocol 1

	Volume per reaction (µl)
RNAse-free water	11.1
2019-nCoV MOM	6
5X Real-time One-step Buffer	6
Real-time One-step Enzyme	2.4
Internal Control	1.5
Sample	3

RSP Protocol 2

	Volume per reaction (µl)
RNAse-free water	12.1
2019-nCoV MOM	6
5X Real-time One-step Buffer	6
Real-time One-step Enzyme	2.4
Internal Control	1.5
Sample	2

Sample 101	19.63	21.64	21.41	21.41	19.24	21.29	20.97	Not detected	not done	not done	not done	not done	9.97	22.38		
Sample 102	17.94	20.22	20.38	20.38	17.85	19.8	20.19	Not detected	not done	not done	not done	not done	9.14	22.34		
Sample 103	23.21	24.57	25.89	25.89	22.64	23.75	25.17	30.55	not done	not done	not done	not done	12.06	20.43		
Sample 104	23.26	25.13	25.09	25.09	22.95	24.55	24.7	Not detected	not done	not done	not done	not done	12.95	20.33		
Sample 105	28.46	31.14	30.16	30.16	28.05	30.62	29.94	33	not done	not done	not done	not done	17.69	17.57		
Sample 105 (inter-run repeat 1)	27.83	31.3	29.61	Not detected	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done		
Sample 105 (inter-run repeat 2)	27.51	31.13	29.64	34.17	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done		
Sample 105 (inter-run repeat 3)	27.42	30.9	29.59	34.35	not done	not done	not done	not done	not done	not done	not done	not done	not done	not done		
Sample 106	31.25	33.8	32.98	29.98	31.26	32.63	32.29	29.95	not done	not done	not done	not done	23.78	17.73		
Sample 107	30.18	33.4	32.09	Not detected	29.1	32	30.97	Not detected	not done	not done	not done	not done	17.43	17.22		
Sample 108	34.64	38.69	35.72	29.7	Not detected	Not detected	Not detected	30.07	not done	not done	not done	not done	29.08	17.91		
Sample 109	Not detected	Not detected	Not detected	25.53	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.9	not done	not done		
Sample 110	Not detected	Not detected	Not detected	25.33	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.2	not done	not done		
Sample 111	Not detected	Not detected	Not detected	24.8	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.2	not done	not done		
Sample 112	Not detected	Not detected	Not detected	27.11	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.7	not done	not done		
Sample 113	Not detected	Not detected	Not detected	25.67	not done	not done	not done	not done	Not detected	Not detected	Not detected	28.8	not done	not done		
Sample 114	Not detected	Not detected	Not detected	27.25	not done	not done	not done	not done	Not detected	Not detected	Not detected	27.8	not done	not done		
Sample 115	Not detected	Not detected	Not detected	24.89	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.5	not done	not done		
Sample 116	Not detected	Not detected	Not detected	24.95	not done	not done	not done	not done	Not detected	Not detected	Not detected	28.2	not done	not done		
Sample 117	Not detected	Not detected	Not detected	26.38	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.1	not done	not done		
Sample 118	Not detected	Not detected	Not detected	25.32	not done	not done	not done	not done	Not detected	Not detected	Not detected	30.1	not done	not done		
Sample 119	Not detected	Not detected	Not detected	24.79	not done	not done	not done	not done	Not detected	Not detected	Not detected	27.2	not done	not done		
Sample 120	Not detected	Not detected	Not detected	24.95	not done	not done	not done	not done	Not detected	Not detected	Not detected	25.1	not done	not done		
Sample 121	Not detected	Not detected	Not detected	26.79	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.3	not done	not done		
Sample 122	Not detected	Not detected	Not detected	26.16	not done	not done	not done	not done	Not detected	Not detected	Not detected	30	not done	not done		
Sample 123	Not detected	Not detected	Not detected	24.67	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.2	not done	not done		
Sample 124	Not detected	Not detected	Not detected	27.42	not done	not done	not done	not done	Not detected	Not detected	Not detected	31.5	not done	not done		
Sample 125	Not detected	Not detected	Not detected	25.78	not done	not done	not done	not done	Not detected	Not detected	Not detected	30.1	not done	not done		
Sample 126	Not detected	Not detected	Not detected	25.39	not done	not done	not done	not done	Not detected	Not detected	Not detected	26.1	not done	not done		
Sample 127	Not detected	Not detected	Not detected	25.24	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.4	not done	not done		
Sample 128	Not detected	Not detected	Not detected	26.19	not done	not done	not done	not done	Not detected	Not detected	Not detected	27.8	not done	not done		
Sample 129	Not detected	Not detected	Not detected	25.1	not done	not done	not done	not done	Not detected	Not detected	Not detected	29.9	not done	not done		
Sample 130	Not detected	Not detected	Not detected	27.16	not done	not done	not done	not done	Not detected	Not detected	Not detected	31	not done	not done		
Sample 131	Not detected	Not detected	Not detected	25.45	not done	not done	not done	not done	Not detected	Not detected	Not detected	28.3	not done	not done		
Sample 132	Not detected	Not detected	Not detected	26.46	not done	not done	not done	not done	Not detected	Not detected	Not detected	26.1	not done	not done		
Sample 133	Not detected	Not detected	Not detected	32.26	Not detected	Not detected	Not detected	27.36	Not done	Not done	Not done	Not done	Not detected	17.12		
Sample 134	Not detected	Not detected	Not detected	31.89	Not detected	Not detected	Not detected	25.91	Not done	Not done	Not done	Not done	Not detected	17.42		
Sample 135	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	30.64	Not done	Not done	Not done	Not done	Not detected	17.19		
Sample 136	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	28.33	Not done	Not done	Not done	Not done	Not detected	17.95		
Excluded 2	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.94		
Sample 137	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	29.41	Not done	Not done	Not done	Not done	Not detected	17.2		
Sample 138	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	28.59	Not done	Not done	Not done	Not done	Not detected	17.22		
Sample 139	Not detected	Not detected	Not detected	32.89	Not detected	Not detected	Not detected	27.13	Not done	Not done	Not done	Not done	Not detected	17.61		
Sample 140	Not detected	Not detected	Not detected	29.6	Not detected	Not detected	Not detected	26.23	Not done	Not done	Not done	Not done	Not detected	17.66		
Sample 141	Not detected	Not detected	Not detected	37.26	Not detected	Not detected	Not detected	29.01	Not done	Not done	Not done	Not done	Not detected	17.33		
Sample 142	Not detected	Not detected	Not detected	33.73	Not detected	Not detected	Not detected	27.16	Not done	Not done	Not done	Not done	Not detected	17.24		
Sample 143	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	29.09	Not done	Not done	Not done	Not done	Not detected	17.39		
Sample 144	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	28.43	Not done	Not done	Not done	Not done	Not detected	17.65		
Sample 145	Not detected	Not detected	Not detected	30.21	Not detected	Not detected	Not detected	26.54	Not done	Not done	Not done	Not done	Not detected	18.2		
Sample 146	Not detected	Not detected	Not detected	30.15	Not detected	Not detected	Not detected	26.42	Not done	Not done	Not done	Not done	Not detected	17.61		
Sample 147	Not detected	Not detected	Not detected	29.81	Not detected	Not detected	Not detected	26.22	Not done	Not done	Not done	Not done	Not detected	17.74		
Sample 148	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	35.28	Not done	Not done	Not done	Not done	Not detected	17.09		
Sample 149	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	28.12	Not done	Not done	Not done	Not done	Not detected	17.48		
Sample 150	Not detected	Not detected	Not detected	30.89	Not detected	Not detected	Not detected	27.06	Not done	Not done	Not done	Not done	Not detected	17.17		
Sample 151	Not detected	Not detected	Not detected	30.83	Not detected	Not detected	Not detected	26.75	Not done	Not done	Not done	Not done	Not detected	17.79		
Sample 152	Not detected	Not detected	Not detected	33.56	Not detected	Not detected	Not detected	27.33	Not done	Not done	Not done	Not done	Not detected	17.63		
Sample 153	Not detected	Not detected	Not detected	38.48	Not detected	Not detected	Not detected	27.53	Not done	Not done	Not done	Not done	Not detected	17.51		
Sample 154	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	29.52	Not done	Not done	Not done	Not done	Not detected	17.14		
Sample 155	Not detected	Not detected	Not detected	31.81	Not detected	Not detected	Not detected	27.17	Not done	Not done	Not done	Not done	Not detected	17.2		
Sample 156	Not detected	Not detected	Not detected	29.44	Not detected	Not detected	Not detected	26.37	Not done	Not done	Not done	Not done	Not detected	17.63		
Sample 157	Not detected	Not detected	Not detected	30.17	Not detected	Not detected	Not detected	26.78	Not done	Not done	Not done	Not done	Not detected	18.39		
Sample 158	Not detected	Not detected	Not detected	29.79	Not detected	Not detected	Not detected	26.39	Not done	Not done	Not done	Not done	Not detected	17.54		
Sample 159	Not detected	Not detected	Not detected	31.82	Not detected	Not detected	Not detected	27.61	Not done	Not done	Not done	Not done	Not detected	17.53		
Sample 160	Not detected	Not detected	Not detected	29.85	Not detected	Not detected	Not detected	26.57	Not done	Not done	Not done	Not done	Not detected	17.45		
Sample 161	Not detected	Not detected	Not detected	30.79	Not detected	Not detected	Not detected	26.93	Not done	Not done	Not done	Not done	Not detected	17.69		
Sample 162	Not detected	Not detected	Not detected	30.83	Not detected	Not detected	Not detected	30.18	Not done	Not done	Not done	Not done	Not detected	17.65		
Excluded 3	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.46		
Excluded 4	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.64		
Excluded 5	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.48		
Sample 163	Not detected	Not detected	Not detected	31.11	Not detected	Not detected	Not detected	33.27	Not done	Not done	Not done	Not done	Not detected	17.5		
Sample 164	Not detected	Not detected	Not detected	31.41	Not detected	Not detected	Not detected	31.13	Not done	Not done	Not done	Not done	Not detected	17.35		
Sample 165	Not detected	Not detected	Not detected	35.23	Not detected	Not detected	Not detected	31.72	Not done	Not done	Not done	Not done	Not detected	17.46		
Excluded 6	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.11		
Sample 166	Not detected	Not detected	Not detected	32.55	Not detected	Not detected	Not detected	32.08	Not done	Not done	Not done	Not done	Not detected	17.91		
Sample 167	Not detected	Not detected	Not detected	31.29	Not detected	Not detected	Not detected	29.91	Not done	Not done	Not done	Not done	Not detected	17.81		
Sample 168	Invalid	Invalid	Invalid	Not detected	Not detected	Not detected	Not detected	37.74	Not done	Not done	Not done	Not done	Not detected	17.43		
Sample 169	Not detected	Not detected	Not detected	30.47	Not detected	Not detected	Not detected	30.18	Not done	Not done	Not done	Not done	Not detected	17.67		
Sample 170	Not detected	Not detected	Not detected	31.71	Not detected	Not detected	Not detected	31.02	Not done	Not done	Not done	Not done	Not detected	17.5		
Sample 171	Not detected	Not detected	Not detected	30.88	Not detected	Not detected	Not detected	30.62	Not done	Not done	Not done	Not done	Not detected	18.03		
Sample 172	Not detected	Not detected	Not detected	33.78	Not detected	Not detected	Not detected	31	Not done	Not done	Not done	Not done	Not detected	17.3		
Sample 173	Not detected	Not detected	Not detected	31.8	Not detected	Not detected	Not detected	31.62	Not done	Not done	Not done	Not done	Not detected	17.31		
Sample 174	Not detected	Not detected	Not detected	31.07	Not detected	Not detected	Not detected	33.54	Not done	Not done	Not done	Not done	Not detected	17.39		
Excluded 7	Invalid	Invalid	Invalid	Not detected	Invalid	Invalid	Invalid	Not detected	Not done	Not done	Not done	Not done	Not detected	17.18		
Sample 175	Not detected	Not detected	Not detected	29.94	Not detected	Not detected	Not detected	32.02	Not done	Not done	Not done	Not done	Not detected	17.47		
Sample 176	Not detected	Not detected	Not detected	30.28	Not detected	Not detected	Not detected	30.15	Not done	Not done	Not done	Not done	Not detected	17.75		
Sample 177	Not detected	Not detected	Not detected	37.16	Not detected	Not detected	Not detected	35.13	Not done	Not done	Not done	Not done	Not detected	17.38		
Sample 178	Not detected	Not detected	Not detected	30.95	Not detected	Not detected	Not detected	30.63	Not done	Not done	Not done	Not done	Not detected	17.4		
Sample 179	Not detected	Not detected	Not detected	32.36	Not detected	Not detected	Not detected	31.02	Not done	Not done	Not done	Not done	Not detected	17.34		
Sample 180	Not detected	Not detected	Not detected	31.14	Not detected	Not detected	Not detected	30.9	Not done	Not done	Not done	Not done	Not detected	17.58		
Sample 181 (replicate 1)	30.35	32.92	31.5	29.34	not done	not done	not done	not done	27.73		29.53	30.04	26.2	not done	not done	
Sample 181 (replicate 2)	30.29	33.01	31.08	29.54	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 181 (replicate 3)	30.63	32.77	31.63	29.48	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 182 (replicate 1)	34.29	34.89	35.43	29.02	not done	not done	not done	not done	30.27		31.9	33.08	26.3	not done	not done	
Sample 182 (replicate 2)	37.58	36.1	37.65	29.28	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 182 (replicate 3)	35.12	Not detected	37.32	29.01	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 183 (replicate 1)	30.44	32.99	31.68	30.6	not done	not done	not done	not done	26.35		27.66	28.9	26.9	not done	not done	
Sample 183 (replicate 2)	30.37	32.83	31.76	31.44	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 183 (replicate 3)	30.19	32.52	31.3	31.86	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 184 (replicate 1)	31.07	33.52	33.62	29.63	not done	not done	not done	not done	27.87		29.7	30.52	29.9	not done	not done	
Sample 184 (replicate 2)	31.07	33.86	32.89	29.66	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 184 (replicate 3)	31.35	32.81	32.93	29.42	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 185 (replicate 1)	31.72	33.37	33.97	29.87	not done	not done	not done	not done	28.26		29.88	31.96	29.1	not done	not done	
Sample 185 (replicate 2)	31.64	33.28	33.5	29.71	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 185 (replicate 3)	31.04	33.79	33.09	30.52	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 186 (replicate 1)	32.74	34.45	34.1	31.05	not done	not done	not done	not done	29.38		30.73	31.6	29.5	not done	not done	
Sample 186 (replicate 2)	32.1	35.44	33.18	33.69	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 186 (replicate 3)	31.85	36.88	33.65	30.71	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 187 (replicate 1)	31.9	35.06	32.88	32.7	not done	not done	not done	not done	28.69		30.01	31.13	26.6	not done	not done	
Sample 187 (replicate 2)	31.26	33.66	32.1	29.34	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 187 (replicate 3)	30.23	33.63	32.23	29.7	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 188 (replicate 1)	33.55	38.11	35.02	30.66	not done	not done	not done	not done	29.68		31.1	32.7	27.1	not done	not done	
Sample 188 (replicate 2)	32.49	34.97	34.78	30.68	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	
Sample 188 (replicate 3)	31.97	35.92	34.97	30.19	not done	not done	not done	not done	Not done		Not done	Not done	Not done	not done	Not done	

Analytical sensitivity determination	Seegene Allplex 2019-nCoV assay with RSP			
Dilution	E gene Ct value	RdRp gene Ct value	N gene Ct value	Internal control
1:320	Not detected	Not detected	Not detected	29.01
1:320	Not detected	Not detected	Not detected	29.73
1:320	Not detected	Not detected	Not detected	29.26
1:320	Not detected	Not detected	Not detected	29.5
1:320	Not detected	37.7	38.33	29.71
1:320	Not detected	Not detected	Not detected	29.89
1:320	Not detected	Not detected	Not detected	29.51
1:320	Not detected	Not detected	39.14	29.76
1:320	37.15	38.47	Not detected	29.4
1:320	Not detected	Not detected	Not detected	29.81
1:320	Not detected	Not detected	38.11	29.47
1:320	36.31	37.39	Not detected	29.75
1:320	Not detected	Not detected	Not detected	29.21
1:320	Not detected	Not detected	Not detected	29.46
1:320	Not detected	Not detected	37.14	29.67
1:320	Not detected	Not detected	Not detected	29.76
1:320	Not detected	Not detected	Not detected	29.79
1:320	Not detected	Not detected	Not detected	29.45
1:320	36.42	Not detected	38.05	29.51
1:320	Not detected	Not detected	Not detected	30.87
1:320	37.76	39.4	39.16	30.99
1:320	Not detected	Not detected	Not detected	30.84
1:320	Not detected	Not detected	Not detected	30.93
1:320	Not detected	Not detected	Not detected	29.89
1:160	Not detected	Not detected	Not detected	29.05
1:160	Not detected	Not detected	Not detected	29.44
1:160	36.45	Not detected	38.94	29.2
1:160	34.84	Not detected	36.41	29.24
1:160	35.67	Not detected	35.44	29.27
1:160	36.77	38.03	Not detected	29.01
1:160	Not detected	Not detected	Not detected	29.91
1:160	Not detected	Not detected	Not detected	29.53
1:160	Not detected	Not detected	36.82	28.93
1:160	Not detected	Not detected	38.32	28.67
1:160	Not detected	Not detected	Not detected	29.02
1:160	Not detected	37.07	36.46	28.59
1:160	Not detected	Not detected	Not detected	29.07
1:160	Not detected	Not detected	Not detected	29.11
1:160	Not detected	Not detected	Not detected	28.93
1:160	Not detected	37.31	38.17	29.31
1:160	Not detected	Not detected	Not detected	29.32
1:160	Not detected	39.62	37.96	28.84
1:160	Not detected	Not detected	Not detected	29.35
1:160	Not detected	36.17	37.1	28.75
1:160	Not detected	Not detected	Not detected	28.27
1:160	Not detected	Not detected	Not detected	28.1
1:160	Not detected	Not detected	Not detected	28.48
1:160	Not detected	Not detected	Not detected	28.29
1:120	Not detected	Not detected	39.35	27.44
1:120	Not detected	Not detected	Not detected	27.86
1:120	36.77	Not detected	38.01	27.58
1:120	Not detected	38.27	38.6	27.73
1:120	Not detected	Not detected	Not detected	27.77
1:120	Not detected	Not detected	Not detected	27.61
1:120	Not detected	38.19	Not detected	28.23

Analytical sensitivity determination	Seegene Allplex 2019-nCoV assay NucliSENS® easyMag® nucleic acid purification			
Dilution	E gene Ct value	RdRp gene Ct value	N gene Ct value	Internal control
1:5000	Not detected	Not detected	Not detected	25.53
1:5000	36.64	Not detected	38.80	25.88
1:5000	Not detected	Not detected	Not detected	25.73
1:5000	Not detected	Not detected	Not detected	25.67
1:5000	36.76	Not detected	Not detected	27.41
1:5000	Not detected	Not detected	38.71	27.29
1:5000	Not detected	Not detected	Not detected	27.44
1:5000	Not detected	Not detected	Not detected	27.48
1:5000	Not detected	Not detected	Not detected	29.66
1:5000	Not detected	Not detected	Not detected	28.03
1:2000	Not detected	Not detected	39.39	26.44
1:2000	Not detected	Not detected	Not detected	25.96
1:2000	Not detected	Not detected	Not detected	26.02
1:2000	35.44	Not detected	Not detected	25.77
1:2000	37.05	38.10	Not detected	25.68
1:2000	Not detected	Not detected	38.00	25.96
1:2000	Not detected	Not detected	Not detected	25.96
1:2000	Not detected	Not detected	Not detected	25.71
1:2000	35.98	37.49	38.87	25.75
1:2000	36.69	Not detected	Not detected	25.75
1:625	Not detected	37.91	Not detected	29.99
1:625	Not detected	Not detected	Not detected	29.74
1:625	35.56	Not detected	38.83	29.62
1:625	Not detected	Not detected	Not detected	29.84
1:625	36.67	Not detected	38.08	29.66
1:625	Not detected	Not detected	Not detected	29.82
1:625	36.05	Not detected	39.11	29.75
1:625	Not detected	Not detected	38.41	29.86
1:625	35.23	38.54	39.16	29.75
1:625	Not detected	36.77	38.6	29.61
1:500	35.98	Not detected	Not detected	27.37
1:500	35.71	Not detected	36.58	26.53
1:500	Not detected	37.24	Not detected	26.41
1:500	36.61	37.10	37.74	26.36
1:500	36.62	Not detected	Not detected	26.29
1:500	Not detected	38.15	37.72	26.39
1:500	Not detected	37.02	38.54	27.02
1:500	Not detected	Not detected	Not detected	26.6
1:500	35.53	Not detected	Not detected	26.56
1:500	36.02	Not detected	Not detected	26.11
1:400	Not detected	Not detected	37.70	25.7
1:400	35.67	37.19	Not detected	25.22
1:400	Not detected	Not detected	37.84	25.16
1:400	Not detected	36.76	37.81	25.27
1:400	Not detected	36.18	38.83	25.29
1:400	37.08	36.79	Not detected	25.49
1:400	Not detected	Not detected	37.74	25.26
1:400	36.01	Not detected	37.60	25.42
1:400	Not detected	37.16	Not detected	25.53
1:400	35.45	Not detected	36.35	25.18
1:200	35.22	Not detected	Not detected	25.92
1:200	33.85	35.43	Not detected	25.2

1:120	36.86	36.59	37.14	28.28
1:120	Not detected	Not detected	38.53	28.03
1:120	Not detected	Not detected	Not detected	27.72
1:120	Not detected	Not detected	Not detected	27.49
1:120	Not detected	38.45	38.45	27.72
1:120	Not detected	37.44	38.4	27.71
1:120	Not detected	Not detected	38.38	28.22
1:120	Not detected	Not detected	38.54	27.98
1:120	Not detected	37.09	Not detected	27.82
1:120	Not detected	Not detected	Not detected	27.85
1:120	Not detected	Not detected	Not detected	27.58
1:120	Not detected	Not detected	Not detected	27.74
1:120	36.3	Not detected	37.47	27.53
1:120	Not detected	Not detected	38.86	28.83
1:120	Not detected	Not detected	Not detected	27.49
1:120	Not detected	Not detected	Not detected	27.99
1:120	37.65	Not detected	Not detected	28.07
1:80	Not detected	Not detected	Not detected	27.66
1:80	Not detected	Not detected	Not detected	27.89
1:80	36.73	36.83	37.17	27.43
1:80	35.8	Not detected	37.53	28.21
1:80	35.72	Not detected	36.61	27.61
1:80	Not detected	Not detected	38.43	27.54
1:80	Not detected	Not detected	Not detected	27.68
1:80	Not detected	Not detected	37.2	27.87
1:80		37.98	37.43	28
1:80	Not detected	Not detected	Not detected	27.56
1:80	Not detected	Not detected	Not detected	28.49
1:80	Not detected	Not detected	Not detected	27.58
1:80	Not detected	Not detected	38.3	27.73
1:80	Not detected	37.87	38.42	27.56
1:80	Not detected	Not detected	38.73	27.81
1:80	Not detected	37.6	Not detected	27.85
1:80	36.65	Not detected	38.27	27.89
1:80	Not detected	Not detected	37.64	27.81
1:80	Not detected	Not detected	38.66	27.53
1:80	Not detected	37.97	Not detected	27.8
1:80	Not detected	Not detected	Not detected	27.76
1:80	36.9	36.72	Not detected	27.65
1:80	Not detected	Not detected	38.5	27.62
1:80	36.72	Not detected	Not detected	27.89
1:40	Not detected	Not detected	36.85	30.32
1:40	36.22	Not detected	Not detected	30.22
1:40	34.77	35.27	36.11	30.27
1:40	35.6	35.36	36.84	29.73
1:40	36.76	36.16	36.69	30.41
1:40	36.48	34.83	36.44	30.84
1:40	36	Not detected	35.45	29.57
1:40	35.95	35.53	36.18	30.91
1:40	35.38	37.62	37.07	30.41
1:40	Not detected	Not detected	Not detected	29.92
1:40	34.75	35.3	35.96	29.93
1:40	37.08	Not detected	36.87	30.49
1:40	36.35	Not detected	37.19	30.25
1:40	38.42	37.85	37.82	30
1:40	35.05	37.21	37.03	30.38
1:40	35.04	36.54	36.12	30.44

1:200	34.32	37.01	36.63	25.26
1:200	37.12	36.37	37.95	25.35
1:200	35.54	36.19	37.48	25.41
1:200	36.68	36.72	37.88	25.31
1:200	35.49	36.35	38.84	25.43
1:200	35.38	Not detected	38.05	25.14
1:200	34.84	Not detected	36.54	25.41
1:200	35.84	Not detected	37.81	25.42

1:40	35.14	Not detected	36.7	30.55
1:40	Not detected	Not detected	37.84	31.02
1:40	Not detected	37.38	38.6	29.59
1:40	Not detected	36.68	37.49	30.04
1:40	Not detected	36.52	36.83	30.61
1:40	36.54		36.54	31.02
1:40	36.4	35.58	36.26	31.01
1:40	38.36	38.58	37.81	31.85
1:20	34.39	35.2	35.31	29.18
1:20	34.54	36.19	39.29	28.96
1:20	33.64	35.36	33.85	28.43
1:20	33.74	35.38	36.13	28.9
1:20	34.3	36.72	36.18	29.53
1:20	33.9	35.37	36.56	28.99
1:20	34.9	35.97	36.96	28.83
1:20	34.93	35.9	36.36	28.97
1:20	34.64	35.04	35.76	29.16
1:20	34.22	36.07	35	28.64
1:20	33.8	35.58	35.46	29.55
1:20	Not detected	35.47	35.96	28.9
1:20	34.06	35.2	35.86	29.02
1:20	34.69	35.44	35.98	28.76
1:20	36.39	35.4	37.33	28.93
1:20	34.38	35.2	36.06	29.08
1:20	35.83	35.87	38.95	29.04
1:20	34.28	35.13	35.89	29.41
1:20	35.09	37.09	36.19	29.29
1:20	34.6	35.58	36.2	29.04
1:20	33.07	35.35	34.85	28.66
1:20	35.33	36.22	38.21	28.67
1:20	36.01	35.91	36.18	28.68
1:20	34.5	34.65	35.64	29.03

Appendix B

Ethics approval letter

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room G50- Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6492
Email: hrec-enquiries@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

29 June 2020

HREC REF:335/2020

Prof D Hardie
Division of Medical Virology
C-18, Laboratory NHLS, NGSH
Email: diana.hardie@uct.ac.za

Dear Prof Hardie

PROJECT TITLE: THE APPLICATION OF A RAPID SAMPLE PREPARATION METHOD FOR SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS-2 MOLECULAR DETECTION IN A SOUTH AFRICAN DIAGNOSTIC LABORATORY-MMED CANDIDATE-DR GERT MARAIS

Thank you for submitting your study to the Faculty of Health Sciences Human Research Ethics Committee (HREC) for review.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study, subject Biosafety Committee approval.

This approval is subject to strict adherence to the HREC recommendations regarding research involving human participants during COVID -19, dated 17 March 2020.

Approval is granted for one year until the 30 June 2021.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: Dr Gert Marais will also be involved in this study.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator must obtain appropriate institutional approval, where necessary, before the research may occur.

Please quote the HREC reference number in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Appendix C

Peer review history

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

Gert Marais , Michelle Naidoo, Nei-yuan Hsiao, Ziyaad Valley-Omar, Heidi Smuts, Diana Hardie

Published: October 20, 2020 • https://doi.org/10.1371/journal.pone.0241029

Peer Review History

Original Submission	August 3, 2020
Decision Letter - Sylvia Maria Bruisten, Editor	September 1, 2020

PONE-D-20-24160

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

PLOS ONE

Dear Dr. Marais,

Thank you for submitting your manuscript to PLOS ONE. After careful consideration, we feel that it has merit but does not fully meet PLOS ONE's publication criteria as it currently stands. Therefore, we invite you to submit a revised version of the manuscript that addresses the points raised during the review process.

This work is interesting and relevant for the worldwide coronavirus testing.

In addition to the comments of both reviewers, with which I agree, there are a few (minor) comments that I would like to add, to further improve the quality of your manuscript.

Please submit your revised manuscript by Oct 16 2020 11:59PM. If you will need more time than this to complete your revisions, please reply to this message or contact the journal office at plosone@plos.org. When you're ready to submit your revision, log on to https://www.editorialmanager.com/pone/ and select the 'Submissions Needing Revision' folder to locate your manuscript file.

Please include the following items when submitting your revised manuscript:

- A rebuttal letter that responds to each point raised by the academic editor and reviewer(s). You should upload this letter as a separate file labeled 'Response to Reviewers'.
- A marked-up copy of your manuscript that highlights changes made to the original version. You should upload this as a separate file labeled 'Revised Manuscript with Track Changes'.
- An unmarked version of your revised paper without tracked changes. You should upload this as a separate file labeled 'Manuscript'.

If you would like to make changes to your financial disclosure, please include your updated statement in your cover letter. Guidelines for resubmitting your figure files are available below the reviewer comments at the end of this letter.

If applicable, we recommend that you deposit your laboratory protocols in protocols.io to enhance the reproducibility of your results. Protocols.io assigns your protocol its own identifier (DOI) so that it can be cited independently in the future. For instructions see: http://journals.plos.org/plosone/s/submission-guidelines#loc-laboratory-protocols

We look forward to receiving your revised manuscript.

Kind regards,

Sylvia Maria Bruisten, Ph.D

Academic Editor

PLOS ONE

Journal Requirements:

When submitting your revision, we need you to address these additional requirements.

1. Please ensure that your manuscript meets PLOS ONE's style requirements, including those for file naming. The PLOS ONE style templates can be found at

https://journals.plos.org/plosone/s/file?id=wjVg/PLOSOne_formatting_sample_main_body.pdf and

https://journals.plos.org/plosone/s/file?id=ba62/PLOSOne_formatting_sample_title_authors_affiliations.pdf

2. Please include captions for your Supporting Information files at the end of your manuscript, and update any in-text citations to match accordingly. Please see our Supporting Information guidelines for more information: http://journals.plos.org/plosone/s/supporting-information.

Additional Editor Comments (if provided):

1. Table 4 and the paragraph where these data are described (page 12) are not completely clear to me. Samples were serially diluted and tested in several replicates (for example 10 or 24). Testing was however for dilutions 1:20 to 1:160 and 1:320 only performed with the RSP method whereas for all other dilutions it was performed with the NA purification method. This does not allow a direct comparison of the sensitivities of the RSP and NA methods. Why were the dilutions not tested in both ways, for example 12 replicates for each dilution for both RSP and NA?

2. Please avoid starting a sentence with a number (for example in lines 161 and 200). Please rephrase these sentences.

3. Line 212: please remove 'are shown' at the end of the sentence.

[Note: HTML markup is below. Please do not edit.]

Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #1: Yes

Reviewer #2: Yes

****

2. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #1: Yes

Reviewer #2: N/A

****

3. Have the authors made all data underlying the findings in their manuscript fully available?

The PLOS Data policy requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

Reviewer #1: No

Reviewer #2: Yes

****

4. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #1: Yes

Reviewer #2: Yes

****

5. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: This manuscripts presents data evaluating procedures to omit the need of nucleic acaid extraction from clinical NP/OP swab samples prior to performing molecular testing for Sars-CoV-2 detection. All results for extraction free procedures are compared to established extraction method (used as gold standards).

The results demonstrate that the extraction free procedure leads to some loss of analytical sensitivity, in particular for samples harbouring a low viral load (high Ct values). In general Ct values for samples without extraction are higher as compared to extracted samples. This could either be due to a reduced amplification efficiency (or even inhibition) or a smaller equivalent of the clinical sample used as input into the PCR reaction.

Specific questions:

1. It would be relevant to present the Ct values of the internal control of all samples w/wo extraction listed in appendix 1 and 2, as this will give insight in the effect of (leaving out) extraction on PCR efficiency / inhibition.

2. 6 previously negative samples were left out from the analysis because the IC failed (even after repeat testing upon dilution). These samples should not have been left out from the analysis but included in table 3, because the information is very relevant in judging the appropriateness and feasbility of the extraction free protocol : The results demonstrate that PCR inhibition was present in 6/185 samples (3%).

3. Nucleic acid extraction using chaotropic agents (Guanidinium salts) result in virus inactivation (loss of infectivity). The extraction-free protocol is based on a 5 minute incubation at 98C. Did the investigators perform any experiments to study the effect of this temperature treatment on sample infectivity (bio-safety). Samples which are manipulated on a QIAgility liquid handling system, given the ‘open environment’ of such a system that lacks HEPA filtering of exhausted air, should be proven to be non-infectious

4. The authors indicate that automation of the PCR setup process significantly reduced robustness of assay performance by reducing the frequency of invalid results. This is just mentioned in the discussion without supporting data. What is menat by invalid results (PC negative / NC positive / IC negative???) and how are these data used in the manuscript (in particular in the S1 appendix)?

5. In the methods section it is described that PCR setup was don using an liquid handling system whereas in the discussion it is mentioned that manual setup was don for at least part of the experiments (and that this is caused operator dependency in the quality of the results). How did these differences in PCR setup procedures affect the overall results and conclusion on the comparison of extraction free procedures to the gold standard methods?

Reviewer #2: This manuscript by Marais and co-workers describes a rapid automated sample preparation method for the detection of SARS-CoV-2. This information is important as limited availability of general nucleic acid purification reagents have impacted SARS-CoV-2 testing worldwide.

There are a number of issues that need to be addressed:

1) The authors mention (lines 131-134) that if the internal control failed (ct <40) the sample was repeated with less sample input. They mention (lines 172-174) in 6 negative samples this was the case after repeat testing. They do not mention however the percentage of samples overall that failed internal control (ct<40) in the initial analysis. This is important because if this percentage is high it would mean a significant increased workload for retesting.

2) The limited availability of reagents was the main reason for this study. The authors may want to comment on availability of consumables for the QIAgility systems.

3) The authors estimate PPA (lines 193-202) based on the mean difference in Ct values between the Nuclisens and RSP method and adding these numbers to Ct values from previously determined samples. They argue that if this newly calculated Ct value was above 40 the sample would be negative if they had used the RSP method. By doing this the authors assume that the relation between the amount of RNA and the Ct value is linear over the entire range of RNA concentrations. The authors do not show this linear correlation. Especially at high Ct values this correlation is almost never linear and generally very variable. In my opinion this method cannot be used to determine the PPA of the RSP method and the authors should delete this part from the manuscript

4) Since the values from the Abbott M2000 system cannot be compared to the Ct values from the Seegene PCR due to intrinsic different analysis method I fail to see what information is added by figure 2.

5) The authors mention that the loss of analytical sensitivity of at least 8 fold was acceptable for clinical application. It is unclear however which criteria played a role in this consideration.

6) Furthermore they mention that the Seegene assay has an analytical sensitivity of 100 RNA copies/reaction with the nuclisense method (and thus > 800 c/reaction for the RSP method). This analytical sensitivity seems rather low compared to other molecular assays which are in the range of 1-50 (see below refs). This should also be taken into consideration with remark 5)

Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, Bleicker T,

Brünink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MP, Drosten C. Detection of 2019 novel

coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020, Jan;25(3):2000045

van Kasteren PB, van der Veer B, van den Brink S, Wijsman L, de Jonge J, van

den Brandt A, Molenkamp R, Reusken CBEM, Meijer A. Comparison of seven

commercial RT-PCR diagnostic kits for COVID-19. J Clin Virol. 2020

Jul;128:104412. doi: 10.1016/j.jcv.2020.104412

Iglói Z, Leven M, Abdel-Karem Abou-Nouar Z, Weller B, Matheeussen V, Coppens

J, Koopmans M, Molenkamp R. Comparison of commercial realtime reverse

transcription PCR assays for the detection of SARS-CoV-2. J Clin Virol. 2020

Aug;129:104510. doi: 10.1016/j.jcv.2020.104510

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6. PLOS authors have the option to publish the peer review history of their article (what does this mean?). If published, this will include your full peer review and any attached files.

If you choose “no”, your identity will remain anonymous but your review may still be made public.

Do you want your identity to be public for this peer review? For information about this choice, including consent withdrawal, please see our Privacy Policy.

Reviewer #1: No

Reviewer #2: **Yes:** Richard Molenkamp

[NOTE: If reviewer comments were submitted as an attachment file, they will be attached to this email and accessible via the submission site. Please log into your account, locate the manuscript record, and check for the action link "View Attachments". If this link does not appear, there are no attachment files.]

While revising your submission, please upload your figure files to the Preflight Analysis and Conversion Engine (PACE) digital diagnostic tool, https://pacev2.apexcovantage.com/. PACE helps ensure that figures meet PLOS requirements. To use PACE, you must first register as a user. Registration is free. Then, login and navigate to the UPLOAD tab, where you will find detailed instructions on how to use the tool. If you encounter any issues or have any questions when using PACE, please email PLOS at figures@plos.org. Please note that Supporting Information files do not need this step.

https://doi.org/10.1371/journal.pone.0241029.r001

Revision 1

Author Response September 28, 2020

Editor’s comments

Comment:

1. Table 4 and the paragraph where these data are described (page 12) are not completely clear to me. Samples were serially diluted and tested in several replicates (for example 10 or 24). Testing was however for dilutions 1:20 to 1:160 and 1:320 only performed with the RSP method whereas for all other dilutions it was performed with the NA purification method. This does not allow a direct comparison of the sensitivities of the RSP and NA methods. Why were the dilutions not tested in both ways, for example 12 replicates for each dilution for both RSP and NA?

Response:

The table shows the same sample, thus allowing direct comparison, that was serially diluted in the range 1:20 to 1:5000. Due to the expected greater sensitivity of NA purification, it was deemed unnecessary to perform multiple replicates at a dilution of less than 1:200 as all replicates tested at 1:200 and 1:400 were detected. With the RSP method, performing additional replicates at a dilution of greater than 1:320, where 33% of replicates were detected, was deemed unnecessary as the goal was to determine the dilution at which targets would be detected with 95% confidence.

The table thus shows the data that was required to determine the dilution at which a specific sample could be detected with 95% confidence using the RSP method and NA purification. This value could then be compared.

The methods section of the manuscript was revised to clarify the selection of sample dilutions.

Comment:

2. Please avoid starting a sentence with a number (for example in lines 161 and 200). Please rephrase these sentences.

Response:

The manuscript was appropriately revised.

Comment:

3. Line 212: please remove 'are shown' at the end of the sentence.

Response:

The manuscript was appropriately revised.

Reviewer 1 Comments

Comment:

1. It would be relevant to present the Ct values of the internal control of all samples w/wo extraction listed in appendix 1 and 2, as this will give insight in the effect of (leaving out) extraction on PCR efficiency / inhibition.

Response:

The tables presented in the appendixes were updated with the internal control values for each sample tested to provide insight into PCR inhibition and extraction efficiency.

Comment:

2. 6 previously negative samples were left out from the analysis because the IC failed (even after repeat testing upon dilution). These samples should not have been left out from the analysis but included in table 3, because the information is very relevant in judging the appropriateness and feasibility of the extraction free protocol : The results demonstrate that PCR inhibition was present in 6/185 samples (3%).

Response:

The samples which failed testing by the RSP method or could not be tested (3.59%) were excluded from table 3 as the standard testing procedure would designate these samples for retesting by an alternative method. Thus assigning these samples as either false negatives or false positives would be inappropriate as these would not be the results reported by the laboratory. However, the manuscript was revised to more clearly highlight this failure rate.

In terms of a feasibility assessment, we feel the current PPA and NPA values along with a reported failure rate is a more reasonable way of presenting the data than reduction of all data to the PPA and NPA.

Comment:

3. Nucleic acid extraction using chaotropic agents (Guanidinium salts) result in virus inactivation (loss of infectivity). The extraction-free protocol is based on a 5 minute incubation at 98C. Did the investigators perform any experiments to study the effect of this temperature treatment on sample infectivity (bio-safety). Samples which are manipulated on a QIAgility liquid handling system, given the 'open environment' of such a system that lacks HEPA filtering of exhausted air, should be proven to be non-infectious

Response:

The sample infectivity was deemed to be ablated after heat treatment at 98 degrees C for 5 minutes based on available publications. Batéjat et al. (2020) demonstrated inactivation of SARS-CoV-2 after heat treatment at 95°C for 3 minutes. Further, Saknimit et al. (1988) demonstrated heat inactivation of coronaviruses other than SARS-CoV-2 beyond specific quantification after heat treatment at 80°C for 1 minute. This literature is referenced in the revised manuscript.

References:

Batéjat, C., Grassin, Q. and Manuguerra, J.C., 2020. Heat inactivation of the Severe Acute Respiratory Syndrome Coronavirus 2. bioRxiv.

Saknimit, M., Inatsuki, I., Sugiyama, Y. and Yagami, K.I., 1988. Virucidal efficacy of physico-chemical treatments against coronaviruses and parvoviruses of laboratory animals. Experimental animals, 37(3), pp.341-345.

Comment:

4. The authors indicate that automation of the PCR setup process significantly reduced robustness of assay performance by reducing the frequency of invalid results. This is just mentioned in the discussion without supporting data. What is meant by invalid results (PC negative / NC positive / IC negative???) and how are these data used in the manuscript (in particular in the S1 appendix)?

Response:

Invalid results in this context specifically refers to samples that lack both internal control amplification and SARS-CoV-2 target amplification. This definition was more clearly presented in the methods section of the revised manuscript.

Prior to implementation of the automated method, staff shortages were frequent due to rapidly scaling testing demand and intermittent quarantining of staff. Thus staff with minimal molecular experience needed to be trained and staff frequently returned after extended absences. We noticed that these events frequently correlated with an increase in invalid rate but a formal critical assessment of the early pandemic SARS-CoV-2 testing performance of our laboratory is beyond the intended purpose of this work. The anecdotal data of fluctuating invalid rate and operator dependency as a potential aetiology motivated for the initiation of this research.

The manuscript and appendixes were revised to include only data directly involved in the generation of the discussed results. The paragraph discussing the motivation for assay automation was revised to remove specific references to previous assay results and protocols and presented as a general discussion of the events leading to the research.

Comment:

5. In the methods section it is described that PCR setup was don using an liquid handling system whereas in the discussion it is mentioned that manual setup was don for at least part of the experiments (and that this is caused operator dependency in the quality of the results). How did these differences in PCR setup procedures affect the overall results and conclusion on the comparison of extraction free procedures to the gold standard methods?

Response:

No results from the manual set-up of the RSP method, which only occurred for prior version of the method used before the initiation of this research, were included. All data from versions of the RSP method not used in the direct generation of the presented results were removed from the appendixes in the updated manuscript. This was initially included to provide insight into the progression of method development.

Reviewer 2 Comments

Comment:

1) The authors mention (lines 131-134) that if the internal control failed (ct <40) the sample was repeated with less sample input. They mention (lines 172-174) in 6 negative samples this was the case after repeat testing. They do not mention however the percentage of samples overall that failed internal control (ct<40) in the initial analysis. This is important because if this percentage is high it would mean a significant increased workload for retesting.

Response:

The manuscript was revised to more clearly show the assay failure rate and steps taken to produce results when the primary protocol failed to produce a result.

Comment:

2) The limited availability of reagents was the main reason for this study. The authors may want to comment on availability of consumables for the QIAgility systems.

Response:

The availability of QIAgility consumables is discussed in the revised manuscript.

Comment:

3) The authors estimate PPA (lines 193-202) based on the mean difference in Ct values between the Nuclisens and RSP method and adding these numbers to Ct values from previously determined samples. They argue that if this newly calculated Ct value was above 40 the sample would be negative if they had used the RSP method. By doing this the authors assume that the relation between the amount of RNA and the Ct value is linear over the entire range of RNA concentrations. The authors do not show this linear correlation. Especially at high Ct values this correlation is almost never linear and generally very variable. In my opinion this method cannot be used to determine the PPA of the RSP method and the authors should delete this part from the manuscript.

Response:

This part of the manuscript was excluded, as suggested, from the revised manuscript.

Comment:

4) Since the values from the Abbott M2000 system cannot be compared to the Ct values from the Seegene PCR due to intrinsic different analysis method I fail to see what information is added by figure 2.

Response:

While the Abbott RealTime SARS-CoV-2 reported CN values are not directly comparable, they are still based on a real-time PCR cycle threshold value and thus we feel the distribution of values is relevant to the data. If only samples with low CN values were used in the validation, for example, the PPA would likely be greater than that reported.

Additionally, while it would be inappropriate to perform any more in-depth analysis due to the disparate test specifics, for operators of the Abbott RealTime SARS-C0V-2 assay we believe a general impression of relative performance as presented by Figure 2 may be valuable.

Comment:

5) The authors mention that the loss of analytical sensitivity of at least 8 fold was acceptable for clinical application. It is unclear however which criteria played a role in this consideration.

Response:

The primary determinant of acceptability for clinical application of the assay was the PPA and NPA. The analytical sensitivity calculated here allows assessment of the relative contribution of PCR inhibition and sample input volume as the aetiology of differing performance but was not used as the determinant of assay acceptability.

Comment:

6) Furthermore they mention that the Seegene assay has an analytical sensitivity of 100 RNA copies/reaction with the nuclisense method (and thus > 800 c/reaction for the RSP method). This analytical sensitivity seems rather low compared to other molecular assays which are in the range of 1-50 (see below refs). This should also be taken into consideration with remark 5)

Response:

While the Seegene reported analytical sensitivity may be poorer than that of other molecular assays, the PPA and NPA were determined from comparison to both the Seegene and Abbott assays. Further, we did not notice a marked difference in performance of the RSP method compared to NA purification relative to its performance compared to the Abbott system as presented in the appendixes. Additionally, the poorer limit of detection still falls below the reported critical value of 6.63 log10 RNA copies/ml associated with infectivity proposed by van Kampen et al. (2020).

Reference:

van Kampen, J.J., van de Vijver, D.A., Fraaij, P.L., Haagmans, B.L., Lamers, M.M., Okba, N., van den Akker, J.P., Endeman, H., Gommers, D.A., Cornelissen, J.J. and Hoek, R.A., 2020. Shedding of infectious virus in hospitalized patients with coronavirus disease-2019 (COVID-19): duration and key determinants. medRxiv.

Attachments
Attachment
Submitted filename: *Response to Reviewers .docx*

https://doi.org/10.1371/journal.pone.0241029.r002

Decision Letter - Sylvia Maria Bruisten, Editor October 7, 2020

PONE-D-20-24160R1

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

PLOS ONE

Dear Dr. Marais,

Thank you for submitting your manuscript to PLOS ONE. After careful consideration, we feel that it has merit but does not fully meet PLOS ONE's publication criteria as it currently stands. Therefore, we invite you to submit a revised version of the manuscript that addresses the points raised during the review process.

There are two minor points that will further improve the manuscript. (see below).

Please submit your revised manuscript by 20 October 2020. If you will need more time than this to complete your revisions, please reply to this message or contact the journal office at plosone@plos.org. When you're ready to submit your revision, log on to https://www.editorialmanager.com/pone/ and select the 'Submissions Needing Revision' folder to locate your manuscript file.

Please include the following items when submitting your revised manuscript:

- A rebuttal letter that responds to each point raised by the academic editor and reviewer(s). You should upload this letter as a separate file labeled 'Response to Reviewers'.
- A marked-up copy of your manuscript that highlights changes made to the original version. You should upload this as a separate file labeled 'Revised Manuscript with Track Changes'.
- An unmarked version of your revised paper without tracked changes. You should upload this as a separate file labeled 'Manuscript'.

If you would like to make changes to your financial disclosure, please include your updated statement in your cover letter. Guidelines for resubmitting your figure files are available below the reviewer comments at the end of this letter.

If applicable, we recommend that you deposit your laboratory protocols in protocols.io to enhance the reproducibility of your results. Protocols.io assigns your protocol its own identifier (DOI) so that it can be cited independently in the future. For instructions see: http://journals.plos.org/plosone/s/submission-guidelines#loc-laboratory-protocols

We look forward to receiving your revised manuscript.

Kind regards,

Sylvia Maria Bruisten, Ph.D

Academic Editor

PLOS ONE

Additional Editor Comments (if provided):

The revised version shows good improvements in manuscript and supplementary files. Most points were answered to satisfaction.

There are two (minor) points that can still improve the manuscript:

1. Table 2 is redundant since here exactly the same mixture scheme is used as in Table 1, with the difference that only 2 µl input in stead of 3 µl was used (which is compensated for by the water volume). I therefor advise to remove Table 2 and to add in the text after 'with a decreased sample volume' '2 µl in stead of 3 µl' (page 7, line 134).

2. Please replace 'greater' by 'higher' before 'mean Ct value'

[Note: HTML markup is below. Please do not edit.]

Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. If the authors have adequately addressed your comments raised in a previous round of review and you feel that this manuscript is now acceptable for publication, you may indicate that here to bypass the “Comments to the Author” section, enter your conflict of interest statement in the “Confidential to Editor” section, and submit your "Accept" recommendation.

Reviewer #2: All comments have been addressed

****

2. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.

Reviewer #2: Yes

****

3. Has the statistical analysis been performed appropriately and rigorously?

Reviewer #2: Yes

****

4. Have the authors made all data underlying the findings in their manuscript fully available?

The PLOS Data policy requires authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception (please refer to the Data Availability Statement in the manuscript PDF file). The data should be provided as part of the manuscript or its supporting information, or deposited to a public repository. For example, in addition to summary statistics, the data points behind means, medians and variance measures should be available. If there are restrictions on publicly sharing data—e.g. participant privacy or use of data from a third party—those must be specified.

Reviewer #2: Yes

****

5. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.

Reviewer #2: Yes

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6. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #2: (No Response)

****

7. PLOS authors have the option to publish the peer review history of their article (what does this mean?). If published, this will include your full peer review and any attached files.

If you choose “no”, your identity will remain anonymous but your review may still be made public.

Do you want your identity to be public for this peer review? For information about this choice, including consent withdrawal, please see our Privacy Policy.

Reviewer #2: No

[NOTE: If reviewer comments were submitted as an attachment file, they will be attached to this email and accessible via the submission site. Please log into your account, locate the manuscript record, and check for the action link "View Attachments". If this link does not appear, there are no attachment files.]

While revising your submission, please upload your figure files to the Preflight Analysis and Conversion Engine (PACE) digital diagnostic tool, https://pacev2.apexcovantage.com/. PACE helps ensure that figures meet PLOS requirements. To use PACE, you must first register as a user. Registration is free. Then, login and navigate to the UPLOAD tab, where you will find detailed instructions on how to use the tool. If you encounter any issues or have any questions when using PACE, please email PLOS at figures@plos.org. Please note that Supporting Information files do not need this step.

https://doi.org/10.1371/journal.pone.0241029.r003

Revision 2

Decision Letter - Sylvia Maria Bruisten, Editor October 8, 2020

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

PONE-D-20-24160R2

Dear Dr. Marais,

We’re pleased to inform you that your manuscript has been judged scientifically suitable for publication and will be formally accepted for publication once it meets all outstanding technical requirements.

This includes to re-number the Table, after the deletion of Table 2.

Within one week, you’ll receive an e-mail detailing the required amendments. When these have been addressed, you’ll receive a formal acceptance letter and your manuscript will be scheduled for publication.

An invoice for payment will follow shortly after the formal acceptance. To ensure an efficient process, please log into Editorial Manager at http://www.editorialmanager.com/pone/, click the 'Update My Information' link at the top of the page, and double check that your user information is up-to-date. If you have any billing related questions, please contact our Author Billing department directly at authorbilling@plos.org.

If your institution or institutions have a press office, please notify them about your upcoming paper to help maximize its impact. If they’ll be preparing press materials, please inform our press team as soon as possible -- no later than 48 hours after receiving the formal acceptance. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information, please contact onepress@plos.org.

Kind regards,

Sylvia Maria Bruisten, Ph.D

Academic Editor

PLOS ONE

Additional Editor Comments (optional):

The requested last adjustments were made, but the Table numbers were not adjusted after deleting Table 2. This should be done in the final version. Then the manuscript can be fully accepted.

Reviewers' comments:

All adjustments were made, but the Tables need to be numbered correctly.

https://doi.org/10.1371/journal.pone.0241029.r005

Author Response October 7, 2020

Thank you for the review of our manuscript. We have prepared responses to the comments provided in addition to a revised manuscript.

Editor's comments

Comment:

1.Table 2 is redundant since here exactly the same mixture scheme is used as in Table 1, with the difference that only 2 µl input in stead of 3 µl was used (which is compensated for by the water volume). I therefor advise to remove Table 2 and to add in the text after 'with a decreased sample volume' '2 µl in stead of 3 µl' (page 7, line 134).

Response:

The manuscript has been appropriately updated.

Comment:

2. Please replace 'greater' by 'higher' before 'mean Ct value'

Response:

The manuscript has been appropriately updated.

Attachments
Attachment
Submitted filename: *Response to Reviewers .docx*

https://doi.org/10.1371/journal.pone.0241029.r004

Formally Accepted

Acceptance Letter - Sylvia Maria Bruisten, Editor October 12, 2020

PONE-D-20-24160R2

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

Dear Dr. Marais:

I'm pleased to inform you that your manuscript has been deemed suitable for publication in PLOS ONE. Congratulations! Your manuscript is now with our production department.

If your institution or institutions have a press office, please let them know about your upcoming paper now to help maximize its impact. If they'll be preparing press materials, please inform our press team within the next 48 hours. Your manuscript will remain under strict press embargo until 2 pm Eastern Time on the date of publication. For more information please contact onepress@plos.org.

If we can help with anything else, please email us at plosone@plos.org.

Thank you for submitting your work to PLOS ONE and supporting open access.

Kind regards,

PLOS ONE Editorial Office Staff

on behalf of

Dr. Sylvia Maria Bruisten

Academic Editor

PLOS ONE

https://doi.org/10.1371/journal.pone.0241029.r006

Open letter on the publication of peer review reports

PLOS recognizes the benefits of transparency in the peer review process. Therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. Reviewers remain anonymous, unless they choose to reveal their names.

We encourage other journals to join us in this initiative. We hope that our action inspires the community, including researchers, research funders, and research institutions, to recognize the benefits of published peer review reports for all parts of the research system.

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Appendix D

Published manuscript

PLOS ONE

RESEARCH ARTICLE

The implementation of a rapid sample preparation method for the detection of SARS-CoV-2 in a diagnostic laboratory in South Africa

Gert Marais[1,2]*, **Michelle Naidoo**[1,2], **Nei-yuan Hsiao**[1,2], **Ziyaad Valley-Omar**[1,3], **Heidi Smuts**[1,2], **Diana Hardie**[1,2]

1 Division of Medical Virology, University of Cape Town, Cape Town, Western Cape, South Africa, **2** Groote Schuur Hospital Virology Diagnostic Laboratory, National Health Laboratory Service, Cape Town, Western Cape, South Africa, **3** Groote Schuur Hospital Tissue Immunology Diagnostic Laboratory, National Health Laboratory Service, Cape Town, Western Cape, South Africa

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Abstract

The SARS-CoV-2 pandemic has resulted in shortages of both critical reagents for nucleic acid purification and highly trained staff as supply chains are strained by high demand, public health measures and frequent quarantining and isolation of staff. This created the need for alternate workflows with limited reliance on specialised reagents, equipment and staff. We present here the validation and implementation of such a workflow for preparing samples for downstream SARS-CoV-2 RT-PCR using liquid handling robots. The rapid sample preparation technique evaluated, which included sample centrifugation and heating prior to RT-PCR, showed a 97.37% (95% CI: 92.55–99.28%) positive percent agreement and 97.30% (95% CI: 90.67–99.52%) negative percent agreement compared to nucleic acid purification-based testing. This method was subsequently adopted as the primary sample preparation method in the Groote Schuur Hospital Virology Diagnostic Laboratory in Cape Town, South Africa.

Introduction

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), an emergent betacoronavirus, was identified as a novel causative agent of severe pneumonia in Wuhan, China in 2019 [1]. The capacity for person-to-person transmission was soon identified and the ensuing pandemic has caused more than seventeen million cases at the time of submission [2].

Currently, diagnostic testing for SARS-CoV-2 relies on molecular techniques, primarily reverse-transcriptase polymerase chain reaction (RT-PCR), from respiratory specimens [3]. The specialised equipment and reagents required to offer these tests at scale has placed significant strain on worldwide supply chains of reagents. Public health measures put in place in numerous countries, including travel restrictions, have further made planning for sustainable

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service delivery difficult as laboratory stock orders may not be filled on time. These issues motivate for the use of diagnostic workflows that favour locally or readily available reagents to, at least partially, insulate supply chains from fluctuations in global demand and evolving travel limiting public health measures. To address these issues, a number of laboratories have successfully developed alternative sample preparation techniques which limit reagent needs and avoid complex nucleic acid (NA) purification protocols [4–6]. There is also a significant cost saving when the reagent-free direct heating method, as described by Fomsgaard and Rosenstierne [4], is used which will become critical if economic fallout from the pandemic intensifies. Staff shortages in the laboratory are an inevitability as social distancing requirements are implemented in concert with increasing demand for diagnostic testing. SARS-CoV-2 outbreaks in the laboratory environment may also introduce unpredictable shortages of critical staff further limiting the capacity of laboratories to offer predictable test turnaround times. The necessary influx of new staff, who may have limited training or training in a related field, can further compromise the reliability of diagnostic laboratory services as the capacity for oversight and quality control is hindered by rapidly evolving testing demands and workflow instability due to reagent shortages and potentially unreliable testing kits due to limited regulatory oversight [7]. All these factors highlight the need for automated workflows that limit the number of laboratory staff-dependent steps and in particular steps requiring specialised training. Automation further limits human error such as sample switches and cross-contamination and are generally amenable to greater degrees of workflow control due to traceable instrument log files.

A chemical reagent-free heat-based rapid sample preparation and inactivation (RSP) [8, 9] method for downstream SARS-CoV-2 RT-PCR amplification is presented here optimised for use on automated liquid handling robots.

Materials and methods

Ethics

Biological material of human origin was anonymised and all clinical and other personally identifiable data delinked with only study specific sample identifiers used along with sample SARS-CoV-2 assay performance data. Ethics approval for this work was granted by the University of Cape Town Human Research Ethics Committee (HREC reference number: 335/2020).

Sample selection

Nasopharyngeal (NP) and oropharyngeal (OP) swabs sent dry or in saline to the National Health Laboratory Service Virology Diagnostic Laboratory in Groote Schuur Hospital from its standard referral area for SARS-CoV-2 testing were included. Selection of 115 samples, which tested positive, and 80 samples, which tested negative, for SARS-CoV-2 by NA purification-based commercial diagnostic assays in use at the diagnostic laboratory was done for the method validation. Spectrum bias was avoided by selecting consecutive samples that tested positive by standard testing over two discrete intervals of regular laboratory workflow. Samples that tested negative were selected randomly from the same intervals. The diagnostic assays in use were the Abbott RealTime SARS-CoV-2 Assay (Abbott Laboratories, USA) running on the Abbott m2000 RealTime system and the Allplex™ 2019-nCoV assay (Seegene, South Korea). The assays were run as per package insert. The Allplex™ 2019-nCoV assay was performed after sample NA purification using the NucliSENS® easyMag® (bioMérieux, France) as per package insert.

Rapid sample preparation

Standard diagnostic testing sample preparation included placing NP or OP swabs in a 2ml Sarstedt sample tube containing 1.5ml autoclaved 0.9% saline. If both a NP and OP swab or multiple swabs of the same type was received, they were combined in a single tube. The swabs were cut to fit in the tube. The tube was then vortexed for 10 seconds. The saline was used as the sample input for downstream assays after which the tube was stored at 4˚C. Stored tubes from diagnostic samples were available for inclusion in the study.

Selected sample tubes were centrifuged at 16 000 *g* for 5 minutes and 50μl of the supernatant was then pipetted into the wells of a 96-well PCR plate. The PCR wells were capped and the plate incubated on a thermocycler at 98˚C for 5 minutes followed by 4˚C for 2 minutes. The PCR plate was then briefly centrifuged and placed on a dedicated QIAgility (Qiagen, Germany) liquid handling instrument for sample-addition.

RT-PCR after rapid sample preparation

Concurrent with sample preparation, a second dedicated QIAgility instrument was used for Allplex™ 2019-nCoV assay master mix preparation and aliquoting into appropriate 8-well PCR strips (Bio-Rad Laboratories, USA). Following master mix preparation, the PCR strips were transferred to the sample-addition QIAgility instrument. The sample input volume and master mix constituents are shown in Table 1.

After sample addition, the PCR strips were sealed and briefly centrifuged before being loaded on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, USA). The real-time PCR cycling parameters recommended by the Allplex™ 2019-nCoV assay package insert were used unchanged. Real-time data analysis was performed using the 2019-nCoV Viewer for Real time Instruments V3 (Ver 3.18.005.003) software as per the Allplex™ 2019-nCoV assay package insert.

If the internal control (RP-IC) was not detected with a cycle threshold (Ct) value <40 and no SARS-CoV-2 targets were detected, the test was deemed invalid and the primary sample was retested with a decreased sample volume input, 2μl instead of 3μl. The remainder of the protocol was unchanged.

Repeatability and analytical sensitivity

Inter-assay reproducibility was assessed using 8 samples with Envelope (E) gene Ct values ranging between 17.16 and 35.63, which were tested in triplicate 7 days after initial testing. Intra-assay reproducibility was assessed by repeating 16 samples in triplicate. Samples were stored at 4˚C while awaiting repeat testing. To assess relative analytical sensitivity, one sample was selected and serially diluted with saline and tested with multiple replicates at dilutions specifically selected to allow calculation of the analytical sensitivity of the Allplex™ 2019-nCoV

Table 1. RT-PCR reaction preparation.

	Volume per reaction (μl)
RNase-free Water	11.1
2019-nCoV MOM (primer and probe mix)	6
5X Real-time One-step Buffer	6
Real-time One-step Enzyme	2.4
Internal control (RP-IC)	1.5
Sample after centrifugation and heating	3
Total volume	30

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assay after NA purification and RSP. The dilution at which SARS-CoV-2 RNA could be detected with 95% confidence was determined for each method by Probit analysis. The absolute analytical sensitivity of the RSP method was then calculated based on the relative analytical sensitivity compared to NA purification-based detection. The absolute analytical sensitivity for NA purification-based detection is reported in the Allplex™ 2019-nCoV assay package insert.

Statistical analysis and graphics

Data visualisation and statistical analysis, including paired t-tests for comparison of target Ct values, a Fisher's exact test for statistical significance determination of the positive percent agreement (PPA) and negative percent agreement (NPA) with NA extraction-based testing and the Wilson/Brown method for 95% confidence interval determination, was done using GraphPad Prism version 8.4.2 for macOS, GraphPad Software, San Diego, California USA, www.graphpad.com.

Results and discussion

The RSP method validation included 115 serially collected samples which tested positive and 80 randomly selected samples from the same period which tested negative for SARS-CoV-2 by NA purification-based testing. After testing with the RSP method, repeat testing with a decreased sample volume was required for 20 of the 195 (10.26%) samples due to detection of neither SARS-CoV-2 targets nor the internal control. One sample could not be tested using the RSP method due to excessive viscosity from nasopharyngeal swab breakdown. Repeat testing failed to generate a result for 6 samples possibly due to sample-specific PCR inhibition. The Allplex™ 2019-nCoV assay result after RSP correlated with that of NA purification-based testing for 111 positive and 72 negative samples as shown in Table 2. No result could be generated for 7 of 195 (3.59%) samples. Raw data is shown in the S1 Appendix.

The PPA and NPA of the RSP method with NA purification-based testing for SARS-CoV-2 demonstrated a P value of <0.0001. The PPA of the RSP method was 97.37% (95% CI: 92.55–99.28%) and the NPA 97.30% (95% CI: 90.67–99.52%). The 7 samples, for which no result could be generated by RSP due to repeated invalid results or sample unsuitability, were excluded from this analysis as standard laboratory practice designates samples for NA purification-based testing in cases of RSP failure.

The Ct values of individual targets of the Allplex™ 2019-nCoV assay were assessed for samples prepared by NucliSENS® easyMag® NA purification and RSP. The E gene, RNA-dependent RNA-polymerase (RdRp) gene and Nucleocapsid (N) gene targets had Ct values that were significantly different with a P value of <0.0001 (Fig 1). The mean difference in Ct values

Table 2. Contingency table used for positive and negative percent agreement with NA purification-based testing calculation.

	Positive SARS-CoV-2	Negative SARS-CoV-2
	Abbott RealTime SARS-CoV-2 Assay or Seegene Allplex™ 2019-nCoV Assay	Abbott RealTime SARS-CoV-2 Assay or Seegene Allplex™ 2019-nCoV Assay
	NA Purification	NA Purification
Positive SARS-CoV-2, RSP method, Seegene Allplex™ 2019-nCoV Assay	111	2
Negative SARS-CoV-2, RSP method, Seegene Allplex™ 2019-nCoV Assay	3	72

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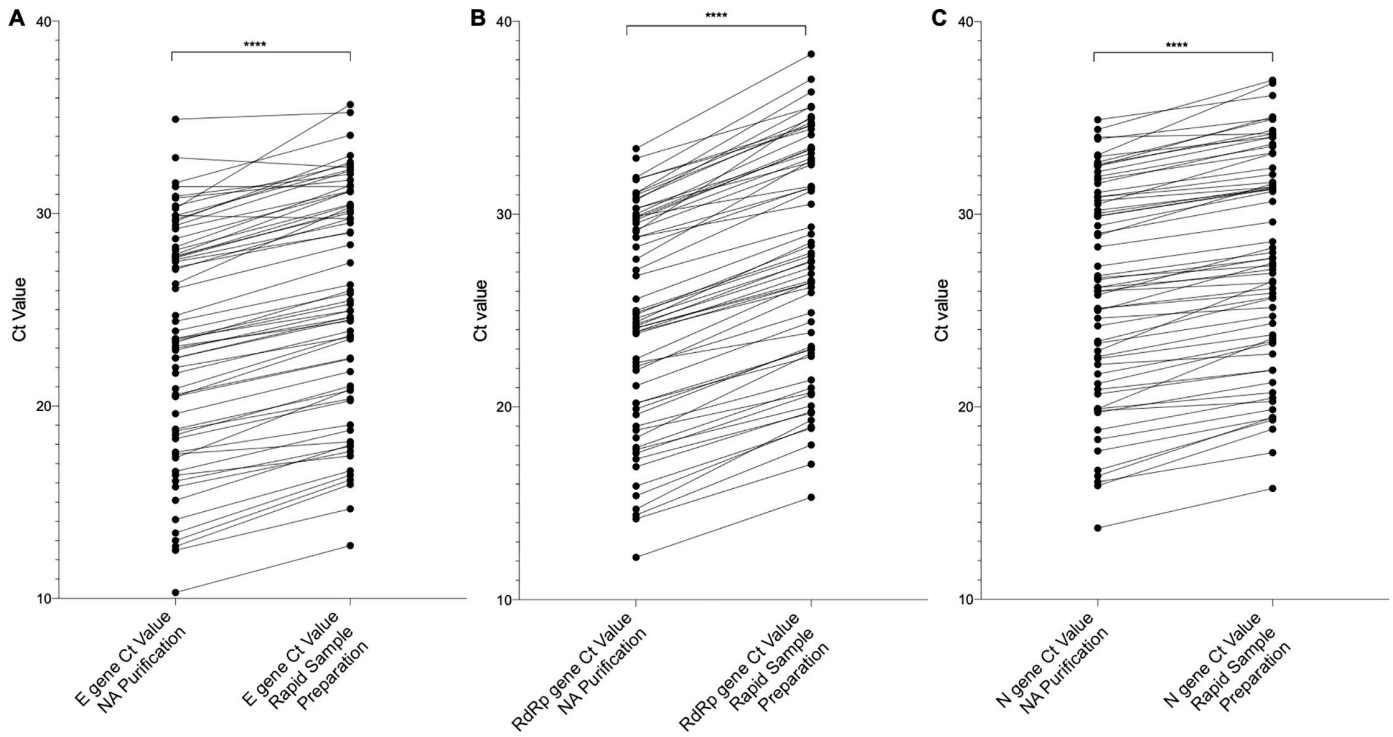

Fig 1. Comparison of target Ct values after RSP and NucliSENS® easyMag NA purification. The Ct values for the SARS-CoV-2 (A) Envelope (E), (B) RNA-dependent RNA-polymerase (RdRp) and (C) Nucleocapsid (N) gene targets are shown for samples tested with the Allplex™ 2019-nCoV assay after NucliSENS® easyMag® NA purification and RSP. The difference in generated Ct values was found to be statistically significant in each case with a P value of <0.0001 as determined by paired t-test.

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between RSP and NA purification was 2.148 (95% CI: 1.909–2.387) for the E gene, 3.271 (95% CI: 3.037–3.506) for the RdRp gene and 1.608 (95% CI: 1.407–1.809) for the N gene, with RSP demonstrating a higher mean Ct value in each case.

The relative performance of the Abbott RealTime SARS-CoV-2 assay and the Allplex™ 2019-nCoV assay after RSP is shown in Fig 2. The Abbott assay reports cycle number (CN) values which are not equivalent to Ct values and thus are not directly comparable.

The single false negative result from the RSP method when compared to NucliSENS® easyMag® NA purification was from a sample that only tested positive for one of the three Allplex™ 2019-nCoV targets, the N gene, with a Ct value of 36.7. The two false negatives from the RSP method when compared to the Abbott RealTime SARS-CoV-2 Assay, which includes NA purification, had high CN values. However, samples with higher CN values were detected thus sample-specific inhibition may also have played a role.

There were two false positive results from the RSP method when compared to the Abbott RealTime SARS-CoV-2 Assay. A single target was detected in both cases with Ct values above 35. This may represent contamination events or the samples may have viral RNA at levels near the limit of detection for both assays. NA contamination in the laboratory is monitored for by frequent testing of environmental swabs and reagent blanks. Multiple negative controls are also included in each run.

The intra-assay repeatability assessment of mean Ct values for the three Allplex™ 2019-nCoV targets showed a coefficient of variance of 1.14%. The inter-assay repeatability assessment of mean Ct values after 7 days of sample storage showed a coefficient of variance of 1.27%.

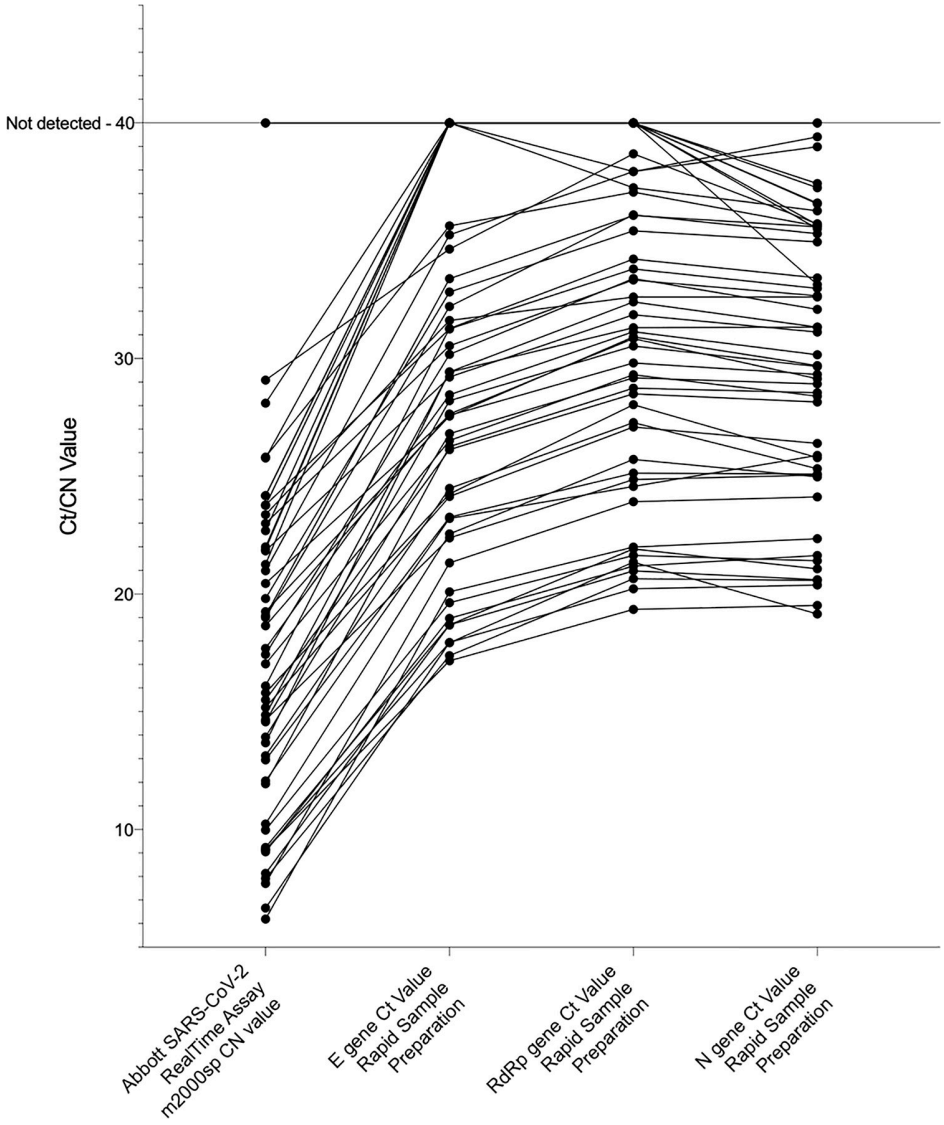

Fig 2. Comparison of target Ct and CN values after RSP and testing with the Abbott RealTime SARS-CoV-2 assay. The Ct values for the SARS-CoV-2 Envelope (E), RNA-dependent RNA-polymerase (RdRp) and Nucleocapsid (N) gene targets are shown for samples tested with the Allplex™ 2019-nCoV assay after RSP and CN values after testing with the Abbott RealTime SARS-CoV-2 assay. A plotted CN or Ct value of 40 indicates that detectable amplification did not occur. The Abbott assay CN values are assay specific and not directly comparable to Ct values, but are shown to demonstrate the performance of the spectrum of selected samples.

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The relative analytical sensitivity of the Allplex™ 2019-nCoV assay after RSP was found to be 807 RNA copies per reaction. This was calculated from the 8.07-fold decrease in analytical sensitivity of the RSP method compared to NucliSENS® easyMag® NA purification-based testing, which has an analytical sensitivity of 100 RNA copies per reaction as per the Allplex™ 2019-nCoV assay package insert. The relative decrease was determined by serially diluting and testing a sample with multiple replicates as shown in Table 3. This relative loss in analytical sensitivity can largely be explained by the smaller sample input volume for RSP. NucliSENS® easyMag® NA purification concentrates sample nucleic acids by a factor of approximately 2,

Table 3. Relative analytical sensitivity assessment.

Dilution	Replicates	Seegene Allplex™ 2019-nCoV Assay	Seegene Allplex™ 2019-nCoV Assay
		RSP Method	NA Purification
		Percentage of Samples Positive	Percentage of Samples Positive
1:20	24	100%	Not done
1:40	24	95.8%	Not done
1:80	24	70.8%	Not done
1:120	24	58.3%	Not done
1:160	24	41.7%	Not done
1:200	10	Not done	100%
1:320	24	33.3%	Not done
1:400	10	Not done	100%
1:500	10	Not done	90%
1:625	10	Not done	70%
1:2000	10	Not done	60%
1:5000	10	Not done	30%

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based on sample input versus elution volume. Additionally, the Allplex™ 2019-nCoV assay input volume after NA purification is 8μl versus the 3μl sample input volume for RSP. Thus, the expected loss in analytical sensitivity would be 5.3-fold which is comparable to the experimentally determined loss of 8.07-fold and suggests that sample inhibition plays a minor role. Raw data is shown in the S2 Appendix.

The performance characteristics were deemed acceptable for clinical diagnostic use in the Groote Schuur Hospital Virology Diagnostic Laboratory and allowed the laboratory to increase the number of samples tested daily by a factor of 5–10 due to the decreased supply chain dependence and simplified workflow. While large quantities of some consumables were still required, such as liquid handling robot tips for the QIAgility instruments, the availability of generic alternatives and the fact that they are neither SARS-CoV-2 specific nor universally required made consumable depletion less of a concern. The reduced processing time further facilitated a more rapid test turnaround time which was beneficial for in-hospital infection control. A stable workflow, not subject to reagent availability dependent variations, also decreased laboratory errors and may allow for improved clinical planning as a result of a stable test turnaround time.

Prior to the automation described in this protocol, earlier versions of the RSP method were susceptible to fluctuating failure rates. This was largely due to human errors arising from staff shortages and rising test volumes. A simple automated workflow was needed to enable staff with minimal molecular experience to be able to perform testing reliably. In particular the time intervals between assay steps and how thoroughly the master mix was mixed prior to aliquoting were identified as sources of assay performance variation. This operator dependency and fluctuating staff availability motivated for the further automation of the process with liquid handling robots and ultimately the validation described here.

The laboratory approach to result interpretation was also affected by the implementation of the RSP method. The approach to NucliSENS® easyMag® NA purification-prepared samples involved release of numerous inconclusive results, despite multiple target amplification at times, due to the known capacity for sample contamination both on the easyMag® instrument and during processing of swabs. The known decrease in sensitivity of the RSP method and the lack of use of the easyMAG® open system for processing, decreased the number of inconclusive results released by our laboratory.

NA purification is the gold-standard in sample processing for RT-PCR, however, in the setting of a pandemic with significant pressures on reagent supply chains and the need for a rapid increase in testing capacity, the RSP method described here presented a reasonable alternative and has been implemented as the primary sample preparation method in the Groote Schuur Hospital Virology Diagnostic Laboratory in South Africa.

Supporting information

S1 Appendix. Sample cycle threshold and cycle number values for SARS-CoV-2 targets and internal controls. The cycle threshold (Ct) and cycle number (CN) values of assay targets and internal controls from the Allplex™ 2019-nCoV and Abbott RealTime SARS-CoV-2 assays respectively are shown for samples used. The mastermix protocol used is also shown. RSP: Rapid sample preparation and inactivation.
(XLSX)

S2 Appendix. Sample cycle threshold values at dilutions used for analytical sensitivity determination. The cycle threshold (Ct) values for the Allplex™ 2019-nCoV assay targets and internal control at dilutions used in the determination of the analytical sensitivity of the rapid sample preparation and inactivation (RSP) method relative to nucleic acid purification.
(XLSX)

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Project administration: Gert Marais.

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Visualization: Gert Marais.

Writing – original draft: Gert Marais.

Writing – review & editing: Michelle Naidoo, Nei-yuan Hsiao, Ziyaad Valley-Omar, Heidi Smuts, Diana Hardie.

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Appendix E

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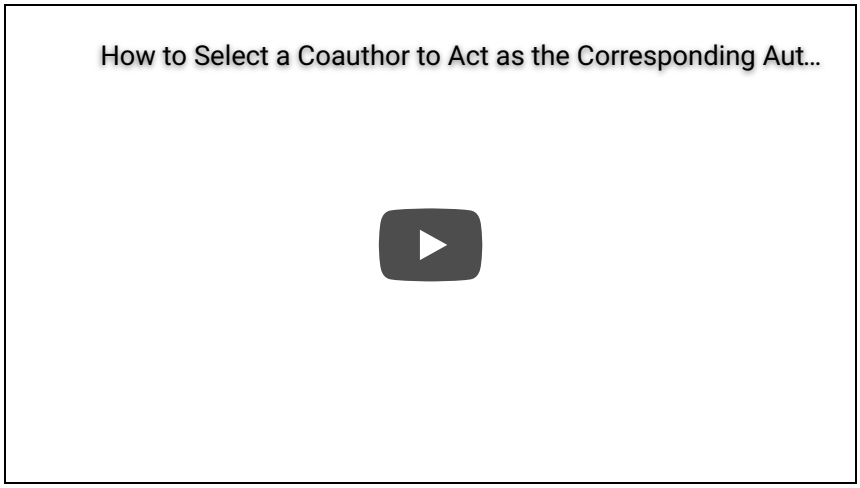

Consortia and group authorship

If a manuscript is submitted on behalf of a consortium or group, include its name in the manuscript byline. Do not add it to the author list in the submission system. You may include the full list of members in the Acknowledgments or in a supporting information file.

PubMed only indexes individual consortium or group author members listed in the article byline. If included, these individuals must qualify for authorship according to our criteria.

Read the group authorship policy.

Author contributions

Provide at minimum one contribution for each author in the submission system. Use the CRediT taxonomy to describe each contribution. Read the policy and the full list of roles.

Contributions will be published with the final article, and they should accurately reflect contributions to the work. The submitting author is responsible for completing this information at submission, and we expect that all authors will have reviewed, discussed, and agreed to their individual contributions ahead of this time.

PLOS ONE will contact all authors by email at submission to ensure that they are aware of the submission.

Cover letter

Upload a cover letter as a separate file in the online system. The length limit is 1 page.

The cover letter should include the following information:

- Summarize the study's contribution to the scientific literature
- Relate the study to previously published work
- Specify the type of article (for example, research article, systematic review, meta-analysis, clinical trial)
- Describe any prior interactions with PLOS regarding the submitted manuscript
- Suggest appropriate Academic Editors to handle your manuscript (see the full list of Academic Editors)
- List any opposed reviewers

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Read about publication fee assistance.

Title page

The title, authors, and affiliations should all be included on a title page as the first page of the manuscript file.

Download our sample title, author list, and affiliations page (PDF)

Abstract

The Abstract comes after the title page in the manuscript file. The abstract text is also entered in a separate field in the submission system.

The Abstract should:

- Describe the main objective(s) of the study
- Explain how the study was done, including any model organisms used, without methodological detail
- Summarize the most important results and their significance
- Not exceed 300 words

Abstracts should not include:

- Citations
- Abbreviations, if possible

Introduction

The introduction should:

- Provide background that puts the manuscript into context and allows readers outside the field to understand the purpose and significance of the study
- Define the problem addressed and why it is important
- Include a brief review of the key literature
- Note any relevant controversies or disagreements in the field
- Conclude with a brief statement of the overall aim of the work and a comment about whether that aim was achieved

Materials and Methods

The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

Supporting reproducibility with protocols

To enhance the reproducibility of your results, we recommend and encourage you to make your protocols public. There are several options:

Protocols associated with Research Articles

Protocol documents may be uploaded as Supporting Information or linked from the Methods section of the article. For laboratory protocols, we recommend protocols.io. Include the DOI link in the Methods section of your manuscript using the following format: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]. This allows editors and reviewers to consult the detailed step-by-step protocol when evaluating your manuscript. You can choose to keep the protocol private on the protocols.io platform until your article is published—at which time it will be published automatically.

Protocols published in their own right

PLOS ONE offers two options for publishing stand-alone protocol articles: Lab Protocols that describe reusable methodologies and Study Protocols that describe detailed plans and proposals for research projects. Specific guidelines apply to the submission of Lab Protocol and Study Protocol manuscripts. Read the detailed instructions for submitting Lab Protocols and Study Protocols.

Results, Discussion, Conclusions

These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled “Results and Discussion”) or a mixed Discussion/Conclusions section (commonly labeled “Discussion”). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

Together, these sections should describe the results of the experiments, the interpretation of these results, and the conclusions that can be drawn.

Authors should explain how the results relate to the hypothesis presented as the basis of the study and provide a succinct explanation of the implications of the findings, particularly in relation to previous related studies and potential future directions for research.

PLOS ONE editorial decisions do not rely on perceived significance or impact, so authors should avoid overstating their conclusions. See the *PLOS ONE* Criteria for Publication for more information.

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

PLOS journals publicly acknowledge the indispensable efforts of our editors and reviewers on an annual basis. To ensure equitable recognition and avoid any appearance of partiality, do not include editors or peer reviewers—named or unnamed—in the Acknowledgments.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., “unpublished work,” “data not shown”). Instead, include those data as supplementary material or deposit the data in a publicly available database.
- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)
- Submitted research should not rely upon retracted research. You should avoid citing retracted articles unless you need to discuss retracted work to provide historical context for your submitted research. If it is necessary to discuss retracted work, state the article’s retracted status in your article’s text and reference list.

Ensure that your reference list includes full and current bibliography details for every cited work at the time of your article’s submission (and publication, if accepted). If cited work is corrected, retracted, or marked with an expression of concern before your article is published, and if you feel it is appropriate to cite the work even in light of the post-publication notice, include in your manuscript citations and full references for both the affected article and the post-publication notice. Email the journal office if you have questions.

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., “We used the techniques developed by our colleagues [19] to analyze the data”). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the “Vancouver” style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (Ailuropoda melanoleuca). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.
Accepted, unpublished articles	Same as published articles, but substitute “Forthcoming” for page numbers or DOI.
Online articles	Huynen MMTE, Martens P, Hilderlink HBM. The health impacts of globalisation: a conceptual framework. Global Health. 2005;1: 14. Available from: http://www.globalizationandhealth.com/content/1/1/14
Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity. arXiv:1403.3301v1 [Preprint]. 2014 [cited 2014 March 17]. Available from: https://128.84.21.199/abs/1403.3301v1 Kording KP, Mensh B. Ten simple rules for structuring papers. BioRxiv [Preprint]. 2016 bioRxiv 088278 [posted 2016 Nov 28; revised 2016 Dec 14; revised 2016 Dec 15; cited 2017 Feb 9]: [12 p.]. Available from: https://www.biorxiv.org/content/10.1101/088278v5 doi: 10.1101/088278
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 2014 Jan 29 [Cited 2014 March 17]. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.

Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and repositories (Figshare, arXiv)	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5]. Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

Supporting information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 20 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an “S” and number. For example, “S1 Appendix” and “S2 Appendix,” “S1 Table” and “S2 Table,” and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

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List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Read the supporting information guidelines for more details about submitting supporting information and multimedia files.

Figures and tables

Figures

Do not include figures in the main manuscript file. Each figure must be prepared and submitted as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Read the guidelines for figures and requirements for reporting blot and gel results.

Figure captions

Figure captions must be inserted in the text of the manuscript, immediately following the paragraph in which the figure is first cited (read order). Do not include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

Read more about figure captions.

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Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Read the guidelines for tables.

Statistical reporting

Manuscripts submitted to *PLOS ONE* are expected to report statistical methods in sufficient detail for others to replicate the analysis performed. Ensure that results are rigorously reported in accordance with community standards and that statistical methods employed are appropriate for the study design.

Consult the following resources for additional guidance:

- SAMPL guidelines, for general guidance on statistical reporting
- *PLOS ONE* guidelines, for clinical trials requirements
- *PLOS ONE* guidelines, for systematic review and meta-analysis requirements
- EQUATOR, for specific reporting guidelines for a range of other study types

Reporting of statistical methods

In the methods, include a section on statistical analysis that reports a detailed description of the statistical methods. In this section:

- List the name and version of any software package used, alongside any relevant references
- Describe technical details or procedures required to reproduce the analysis
- Provide the repository identifier for any code used in the analysis (See our code-sharing policy.)

Statistical reporting guidelines:

- Identify research design and independent variables as being between- or within-subjects
- For pre-processed data:
  - Describe any analysis carried out to confirm the data meets the assumptions of the analysis performed (e.g. linearity, co-linearity, normality of the distribution).
  - If data were transformed include this information, with a reason for doing so and a description of the transformation performed
- Provide details of how outliers were treated and your analysis, both with the full dataset and with the outliers removed
- If relevant, describe how missing/excluded data were handled
- Define the threshold for significance (alpha)
- If appropriate, provide sample sizes, along with a description of how they were determined. If a sample size calculation was performed, specify the inputs for power, effect size and alpha. Where relevant, report the number of independent replications for each experiment.
- For analyses of variance (ANOVAs), detail any post hoc tests that were performed

- Include details of any corrections applied to account for multiple comparisons. If corrections were not applied, include a justification for not doing so
- Describe all options for statistical procedures. For example, if t-tests were performed, state whether these were one- or two-tailed. Include details of the type of t-test conducted (e.g. one sample, within-/between-subjects).
- For step-wise multiple regression analyses:
  - Report the alpha level used
  - Discuss whether the variables were assessed for collinearity and interaction
  - Describe the variable selection process by which the final model was developed (e.g., forward-stepwise; best subset). See SAMPL guidelines.
- For Bayesian analysis explain the choice of prior trial probabilities and how they were selected. Markov chain Monte Carlo settings should be reported.

Reporting of statistical results

Results must be rigorously and appropriately reported, in keeping with community standards.

- **Units of measurement.** Clearly define measurement units in all tables and figures.
- **Properties of distribution.** It should be clear from the text which measures of variance (standard deviation, standard error of the mean, confidence intervals) and central tendency (mean, median) are being presented.
- **Regression analyses.** Include the full results of any regression analysis performed as a supplementary file. Include all estimated regression coefficients, their standard error, p-values, and confidence intervals, as well as the measures of goodness of fit.
- **Reporting parameters.** Test statistics (F/t/r) and associated degrees of freedom should be provided. Effect sizes and confidence intervals should be reported where appropriate. If percentages are provided, the numerator and denominator should also be given.
- **P-values.** Report exact p-values for all values greater than or equal to 0.001. P-values less than 0.001 may be expressed as $p < 0.001$, or as exponentials in studies of genetic associations.
- **Displaying data in plots.** Format plots so that they accurately depict the sample distribution. 3D effects in plots can bias and hinder interpretation of values, so avoid them in cases where regular plots are sufficient to display the data.
- **Open data.** As explained in PLOS's Data Policy, be sure to make individual data points, underlying graphs and summary statistics available at the time of publication. Data can be deposited in a repository or included within the Supporting Information files.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

See instructions on providing underlying data to support blot and gel results.

Read our policy on data availability.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

See our list of recommended repositories.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.

- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at full submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Striking image

You can choose to upload a “Striking Image” that we may use to represent your article online in places like the journal homepage or in search results.

The striking image must be derived from a figure or supporting information file from the submission, i.e., a cropped portion of an image or the entire image. Striking images should ideally be high resolution, eye-catching, single panel images, and should ideally avoid containing added details such as text, scale bars, and arrows.

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Financial Disclosure Statement

This information should describe sources of funding that have supported the work. It is important to gather these details prior to submission because your financial disclosure statement cannot be changed after initial submission without journal approval. If your manuscript is published, your statement will appear in the Funding section of the article.

Enter this statement in the Financial Disclosure section of the submission form. Do not include it in your manuscript file.

The statement should include:

- Specific grant numbers
- Initials of authors who received each award
- Full names of commercial companies that funded the study or authors

- Initials of authors who received salary or other funding from commercial companies
- URLs to sponsors' websites

Also state whether any sponsors or funders (other than the named authors) played any role in:

- Study design
- Data collection and analysis
- Decision to publish
- Preparation of the manuscript

If they had no role in the research, include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript."

If the study was unfunded, include this sentence as the Financial Disclosure statement: "The author(s) received no specific funding for this work."

Read our policy on disclosure of funding sources.

Competing interests

This information should not be in your manuscript file; you will provide it via our submission system.

All potential competing interests must be declared in full. If the submission is related to any patents, patent applications, or products in development or for market, these details, including patent numbers and titles, must be disclosed in full.

Read our policy on competing interests.

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For manuscripts disputing previously published work, it is *PLOS ONE* policy to invite a signed review by the disputed author during the peer review process. This procedure is aimed at ensuring a thorough, transparent, and productive review process.

If the disputed author chooses to submit a review, it must be returned in a timely fashion and contain a full declaration of all competing interests. The Academic Editor will consider any such reviews in light of the competing interest.

Authors submitting manuscripts disputing previous work should explain the relationship between the manuscripts in their cover letter, and will be required to confirm that they accept the conditions of this review policy before the manuscript is considered further.

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Upon submission, authors must confirm that the manuscript, or any related manuscript, is not currently under consideration or accepted elsewhere. If related work has been submitted to *PLOS ONE* or elsewhere, authors must include a copy with the submitted article. Reviewers will be asked to comment on the overlap between related submissions.

We strongly discourage the unnecessary division of related work into separate manuscripts, and we will not consider manuscripts that are divided into "parts." Each submission to *PLOS ONE* must be written as an independent unit and should not rely on any work that has not already been accepted for publication. If related manuscripts are submitted to *PLOS ONE*, the authors may be advised to combine them into a single manuscript at the editor's discretion.

Read our policies on related manuscripts.

Preprints

PLOS encourages authors to post preprints to accelerate the dissemination of research and support authors who wish to share their work early and receive feedback before formal peer review. Deposition of manuscripts with preprint servers does not impact consideration of the manuscript at any PLOS journal.

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Authors submitting manuscripts in the life sciences to *PLOS ONE* may opt-in to post their work on bioRxiv during the *PLOS ONE* initial submission process.

Read more about preprints.

Learn how to post a preprint to bioRxiv during *PLOS ONE* initial submission.

Guidelines for Specific Study Types

Study design, reporting, and analyses are assessed against all relevant research and methodological technique standards held by the community. Guidelines for specific study types are outlined below.

Registered Reports

Submission and format requirements for Registered Report Protocols and Registered Reports are similar to those for a regular submission and may be specific to your study type. For instance, if your Registered Report Protocol submission is about a Clinical Trial or a Systematic Review, follow the appropriate guidelines.

For Registered Report Protocols:

- Provide enough methodological detail to make the study reproducible and replicable
- Confirm that data will be made available upon study completion in keeping with the PLOS Data policy
- Include ethical approval or waivers, if applicable
- Preliminary or pilot data may be included, but only if necessary to support the feasibility of the study or as a proof of principle
- For meta-analyses or Clinical Trials, use the protocol-specific reporting guidelines PRISMA-P or SPIRIT respectively

For more guidance on format and presentation of a protocol, consult the sample template hosted by the Open Science Framework. Discipline-specific and study-specific templates are also available.

If data need to be collected, modified or processed specifically for your study, or if participants need to be recruited specifically for your study, then it should occur only after your Registered Report Protocol is accepted for publication.

For Registered Report Research Articles:

- Report the results of all planned analyses and, if relevant, detail and justify all deviations from the protocol.
- The manuscript may also contain exploratory, unplanned analyses.

Read more about Registered Report framework.

Human subjects research

All research involving human participants must have been approved by the authors' Institutional Review Board (IRB) or by equivalent ethics committee(s), and must have been conducted according to the principles expressed in the Declaration of Helsinki. Authors should be able to submit, upon request, a statement from the IRB or ethics committee indicating approval of the research. We reserve the right to reject work that we believe has not been conducted to a high ethical standard, even when formal approval has been obtained.

Subjects must have been properly instructed and have indicated that they consent to participate by signing the appropriate informed consent paperwork. Authors may be asked to submit a blank, sample copy of a subject consent form. If consent was verbal instead of written, or if consent could not be obtained, the authors must explain the reason in the manuscript, and the use of verbal consent or the lack of consent must have been approved by the IRB or ethics committee.

All efforts should be made to protect patient privacy and anonymity. Identifying information, including photos, should not be included in the manuscript unless the information is crucial and the individual has provided written consent by completing the Consent Form for Publication in a PLOS Journal (PDF). Download additional translations of the form here. More information about patient privacy, anonymity, and informed consent can be found in the International Committee of Medical Journal Editors (ICMJE) Privacy and Confidentiality guidelines.

Manuscripts should conform to the following reporting guidelines:

- Studies of diagnostic accuracy: STARD
- Observational studies: STROBE
- Microarray experiments: MIAME
- Other types of health-related research: Consult the EQUATOR web site for appropriate reporting guidelines

Methods sections of papers on research using human subjects or samples must include ethics statements that specify:

- **The name of the approving institutional review board or equivalent committee(s)**. If approval was not obtained, the authors must provide a detailed statement explaining why it was not needed
- **Whether informed consent was written or oral**. If informed consent was oral, it must be stated in the manuscript:
  - Why written consent could not be obtained
  - That the Institutional Review Board (IRB) approved use of oral consent
  - How oral consent was documented

For studies involving humans categorized by race/ethnicity, age, disease/disabilities, religion, sex/gender, sexual orientation, or other socially constructed groupings, authors should:

- Explicitly describe their methods of categorizing human populations
- Define categories in as much detail as the study protocol allows
- Justify their choices of definitions and categories, including for example whether any rules of human categorization were required by their funding agency
- Explain whether (and if so, how) they controlled for confounding variables such as socioeconomic status, nutrition, environmental exposures, or similar factors in their analysis

In addition, outmoded terms and potentially stigmatizing labels should be changed to more current, acceptable terminology. Examples: “Caucasian” should be changed to “white” or “of [Western] European descent” (as appropriate); “cancer victims” should be changed to “patients with cancer.”

For papers that include identifying, or potentially identifying, information, authors must download the Consent Form for Publication in a PLOS Journal, which the individual, parent, or guardian must sign once they have read the paper and been informed about the terms of PLOS open-access license. The signed consent form should not be submitted with the manuscript, but authors should securely file it in the individual's case notes and the methods section of the manuscript should explicitly state that consent authorization for publication is on file, using wording like:

The individual in this manuscript has given written informed consent (as outlined in PLOS consent form) to publish these case details.

For more information about *PLOS ONE* policies regarding human subjects research, see the Publication Criteria and Editorial Policies.

Clinical trials

Clinical trials are subject to all policies regarding human research. *PLOS ONE* follows the World Health Organization's (WHO) definition of a clinical trial:

> *A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.*

All clinical trials must be registered in one of the publicly-accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors). Authors must provide the trial registration number. Prior disclosure of results on a clinical trial registry site will not affect consideration for publication. We reserve the right to inform authors' institutions or ethics committees, and to reject the manuscript, if we become aware of unregistered trials.

PLOS ONE supports prospective trial registration (i.e. before participant recruitment has begun) as recommended by the ICMJE's clinical trial registration policy. **Where trials were not publicly registered before participant recruitment began**, authors must:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the reason for failing to register before participant recruitment

Clinical trials must be reported according to the relevant reporting guidelines, i.e. CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate. The intervention should be described according to the requirements of the TIDieR checklist and guide. Submissions must also include the study protocol as supporting information, which will be published with the manuscript if accepted.

Authors of manuscripts describing the results of clinical trials must adhere to the CONSORT reporting guidelines appropriate to their trial design, available on the CONSORT Statement web site. Before the paper can enter peer review, authors must:

- The name of the registry and the registration number must be included in the Abstract.
- Provide a copy of the trial protocol as approved by the ethics committee and a completed CONSORT checklist as supporting information (which will be published alongside the paper, if accepted). This should be named S1 CONSORT Checklist.
- Include the CONSORT flow diagram as the manuscript's "Fig 1"

Any deviation from the trial protocol must be explained in the paper. Authors must explicitly discuss informed consent in their paper, and we reserve the right to ask for a copy of the patient consent form.

The name of the registry and the registry number must be provided in the Abstract. If the trial is registered in more than one location, please provide all relevant registry names and numbers.

Lab Protocols

Lab Protocols consist of two interlinked components: a protocol hosted on the protocols.io platform and a peer-reviewed article on *PLOS ONE* that contextualises the protocol.

protocols.io is a secure open access platform that specializes in laboratory protocols. It allows scientists to share, discover and reuse up-to-date protocol knowledge. The platform provides specialist tools and guidance on how to add each element of the protocol, including the title, abstract, steps, files, links, reagents, measurements, formulae, videos, charts and more.

The *PLOS ONE* article component must comply with the general submission guidelines (detailed above in this article).

The *PLOS ONE* article component must also comply with the general *PLOS ONE* criteria for publication and in addition it should:

- Present a step-by-step protocol that adds value to the published literature.
- Provide evidence that the protocol works, by:
  - Linking, in the Introduction section, to at least one supporting peer-reviewed publication in which the protocol was applied to generate data.
    or
  - Providing supporting validation or benchmarking data which demonstrates that the underlying method achieves its intended purpose.
- Link, in the Materials and Methods section, to the protocol.io component, using the digital object identifier (DOI) and format provided by protocols.io, for example *https://dx.doi.org/10.17504/protocols.io[....]*.
- Describe the appropriate controls, sample sizes and replication needed to ensure that the data are robust and reproducible.
- Provide the protocol as a supporting information (S1) file for printing purposes. You can download a PDF from protocols.io for this purpose.

Download a sample Lab Protocol template

Lab Protocols describing routine methods, or extensions or modifications of routine methods, add little or no value to the published literature and will not be considered for publication.

Lab Protocols are subject to the same editorial and peer review process as all other articles, except that the peer review process may be expedited and carried out by one internal Academic Editor and one external reviewer.

Lab Protocols are eligible for both signed and published peer review.

We encourage you to post your protocol to the protocols.io platform before submitting your manuscript to *PLOS ONE*, or at the latest, before the editorial and peer review process. This approach is optional, but beneficial, because:

- Your DOI is assigned on the protocols.io platform. You need this identifier to link out from the Material and Methods section of your manuscript.
- You can keep your protocol private on the protocols.io platform (until you are satisfied that it is ready for publication), but still assign a DOI.
- The protocol will be accessible to editors and reviewers during the editorial and peer review process.

If you prefer to submit your manuscript to *PLOS ONE* before uploading your protocol to protocols.io, please provide your protocol as a supporting information (S1) file. You can use protocols.io's editorial service at no cost: they will check and publish your protocol for you. As part of *PLOS ONE*'s partnership with protocols.io, your waiver code for this purpose will be provided in the first decision letter.

Preprint posting is not available for Lab Protocols and bioRxiv does not accept them.

Study Protocols

Study Protocols describe plans for conducting research projects and consist of a single article on *PLOS ONE*.

Study Protocols must comply with the *PLOS ONE* general submission guidelines (detailed above in this article) and any guidelines specific to the related research study type. In addition, the protocol must:

- Relate to a research study that has not yet generated results.
- Be submitted before recruitment of participants or collection of data for the study is complete.
- Meet the same standards for ethics of experimentation and research integrity as the research study. If it involves human or animal subjects, cell lines or field sampling, or has potential biosafety implications, prior approval from the relevant ethics body must be obtained prior to submission. Please contact us if you have a valid reason for not obtaining approval.

Additional prerequisites apply for these study types:

- Clinical trials:
  - The trial must be registered prior to submission of your protocol in one of the publicly accessible registries approved by the WHO or ICMJE (International Committee of Medical Journal Editors).
  - The name of the registry and the trial or study registration number must be included in the Abstract.
  - A copy of the protocol that was approved by the ethics committee must be submitted as a supplementary information file. Please provide an addtional English translation if the original document is not in English.
  - A SPIRIT schedule of enrollment, interventions, and assessments must be included as the manuscript's Figure 1, and a completed SPIRIT checklist must be uploaded as Supporting Information file S1.
- Systematic reviews and meta-analyses:
  - A completed PRISMA-P checklist must be provided as a supporting information (SI) file. See PRISMA-P Explanation and Elaboration for more information on completing your checklist.

Study Protocols must also comply with general *PLOS ONE* criteria for publication and in addition you should:

- include the word "Protocol" in your Title.
- include a detailed description of the planned study in the Materials and Methods section.This should provide sufficient methodological detail for the protocol to be reproducible and replicable. Your description should cover all relevant and applicable facts and hypothesis, including:
  - the aim, design, and settling
  - the sample size calculation
  - how data saturation will be determined (for qualitative studies)

- the characteristics of participants e.g., inclusion and exclusion criteria, sample selection criteria, variables to be measured, randomization and blinding criteria (where applicable), and how informed consent will be obtained
- how materials will be selected and used e.g., where and how they will be sourced, the processes, interventions, or comparisons to be used, the outcomes to be measured, and when and how they will be measured
- the data management plan
- safety considerations
- the type of data and statistical analyses to be used
- the status and timeline of the study, including whether participant recruitment or data collection has begun
- where and when the data will be made available. See our Data Availability policy for more.

- include an analysis of preliminary or pilot data, only if it is necessary to support the feasibility of the study or as a proof of principle. This is optional.
- we encourage authors you to register with OSF and provide the your registration number in the Materials and Methods section. This is optional.
- optionally add any other SI files, figures or tables that elaborate or authenticate the protocol: e.g., any reporting checklists applicable to your study type.

Read the supporting information guidelines for more details about adding SI files.

Download our sample Study Protocol template or an OSF discipline or study-specific template.

Study Protocols are subject to the same editorial and peer review process as all other articles, and are eligible for both signed and published peer review.

You can expedite the review process by providing:

- proof of external funding. This is typically your funding approval letter and a list of the names and credentials of the funders who conducted the external peer review of the protocol. Include an English translation if needed.
- proof of ethics approval (if required). This is typically the approval or waiver letter from the relevant ethics body and a copy of the protocol approved by this body.

These documents are used for internal purposes and do not form part of the published Study Protocol. Expedited review is conducted by an internal Staff Editor only and bypasses the external review process.

If the Study Protocol describes a replication study or involves re-analysis of published work, we will invite the author of the initial or replicated study to provide a signed review.

We encourage you to share your Study Protocol with other researchers, either before or after submission. You can publish it on your website or protocols.io, or submit it for posting on medRxiv or another preprint server.

Animal research

All research involving vertebrates or cephalopods must have approval from the authors' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee(s), and must have been conducted according to applicable national and international guidelines. Approval must be received prior to beginning research.

Manuscripts reporting animal research must state in the Methods section:

- The full name of the relevant ethics committee that approved the work, and the associated permit number(s).
- Where ethical approval is not required, the manuscript should include a clear statement of this and the reason why. Provide any relevant regulations under which the study is exempt from the requirement for approval.
- Relevant details of steps taken to ameliorate animal suffering.

Example ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Minnesota (Protocol Number: 27-2956). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Authors should always state the organism(s) studied in the Abstract. Where the study may be confused as pertaining to clinical research, authors should also state the animal model in the title.

To maximize reproducibility and potential for re-use of data, we encourage authors to follow the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines for all submissions describing laboratory-based animal research and to upload a completed ARRIVE Guidelines Checklist to be published as supporting information.

Non-human primates

Manuscripts describing research involving non-human primates must report details of husbandry and animal welfare in accordance with the recommendations of the Weatherall report, *The use of non-human primates in research*, including:

- Information about housing, feeding, and environmental enrichment.
- Steps taken to minimize suffering, including use of anesthesia and method of sacrifice, if appropriate.

Random source animals

Manuscripts describing studies that use random source (e.g. Class B dealer-sourced in the USA), shelter, or stray animals will be subject to additional scrutiny and may be rejected if sufficient ethical and scientific justification for the study design is lacking.

Unacceptable euthanasia methods and anesthetic agents

Manuscripts reporting use of a euthanasia method(s) classified as unacceptable by the American Veterinary Medical Association or use of an anesthesia method(s) that is widely prohibited (e.g., chloral hydrate, ether, chloroform) must include at the time of initial submission, scientific justification for use in the specific study design, as well as confirmation of approval for specific use from their animal research ethics committee. These manuscripts may be subject to additional ethics considerations prior to publication.

Humane endpoints

Manuscripts reporting studies in which death of a regulated animal (vertebrate, cephalopod) is a likely outcome or a planned experimental endpoint, must comprehensively report details of study design, rationale for the approach, and methodology, including consideration of humane endpoints. This applies to research that involves, for instance, assessment of survival, toxicity, longevity, terminal disease, or high rates of incidental mortality.

Definition of a humane endpoint

A humane endpoint is a predefined experimental endpoint at which animals are euthanized when they display early markers associated with death or poor prognosis of quality of life, or specific signs of severe suffering or distress. Humane endpoints are used as an alternative to allowing such conditions to continue or progress to death following the experimental intervention ("death as an endpoint"), or only euthanizing animals at the end of an experiment. Before a study begins, researchers define the practical observations or measurements that will be used during the study to recognize a humane endpoint, based on anticipated clinical, physiological, and behavioral signs. Please see the NC3Rs guidelines for more information. Additional discussion of humane endpoints can be found in this article: Nuno H. Franco, Margarida Correia-Neves, I. Anna S. Olsson (2012) How "Humane" Is Your Endpoint? — Refining the Science-Driven Approach for Termination of Animal Studies of Chronic Infection. PLoS Pathog 8(1): e1002399 doi.org/10.1371/journal.ppat.1002399.

Full details of humane endpoints use must be reported for a study to be reproducible and for the results to be accurately interpreted.

For studies in which death of an animal is an outcome or a planned experimental endpoint, authors should include the following information in the Methods section of the manuscript:

- The specific criteria (i.e. humane endpoints) used to determine when animals should be euthanized.

- The duration of the experiment.
- The numbers of animals used, euthanized, and found dead (if any); the cause of death for all animals.
- How frequently animal health and behavior were monitored.
- All animal welfare considerations taken, including efforts to minimize suffering and distress, use of analgesics or anaesthetics, or special housing conditions.

If humane endpoints were not used, the manuscript should report:

- A scientific justification for the study design, including the reasons why humane endpoints could not be used, and discussion of alternatives that were considered.
- Whether the institutional animal ethics committee specifically reviewed and approved the anticipated mortality in the study design.

Observational and field studies

Methods sections for submissions reporting on any type of field study must include ethics statements that specify:

- Permits and approvals obtained for the work, including the full name of the authority that approved the study; if none were required, authors should explain why
- Whether the land accessed is privately owned or protected
- Whether any protected species were sampled
- Full details of animal husbandry, experimentation, and care/welfare, where relevant

Paleontology and archaeology research

Manuscripts reporting paleontology and archaeology research must include descriptions of methods and specimens in sufficient detail to allow the work to be reproduced. Data sets supporting statistical and phylogenetic analyses should be provided, preferably in a format that allows easy re-use. Read the policy.

Specimen numbers and complete repository information, including museum name and geographic location, are required for publication. Locality information should be provided in the manuscript as legally allowable, or a statement should be included giving details of the availability of such information to qualified researchers.

If permits were required for any aspect of the work, details should be given of all permits that were obtained, including the full name of the issuing authority. This should be accompanied by the following statement:

> *All necessary permits were obtained for the described study, which complied with all relevant regulations.*

If no permits were required, please include the following statement:

> *No permits were required for the described study, which complied with all relevant regulations.*

Manuscripts describing paleontology and archaeology research are subject to the following policies:

- **Sharing of data and materials.** Any specimen that is erected as a new species, described, or figured must be deposited in an accessible, permanent repository (i.e., public museum or similar institution). If study conclusions depend on specimens that do not fit these criteria, the article will be rejected under *PLOS ONE*'s data availability criterion.
- **Ethics.** *PLOS ONE* will not publish research on specimens that were obtained without necessary permission or were illegally exported.

Systematic reviews and meta-analyses

A systematic review paper, as defined by The Cochrane Collaboration, is a review of a clearly formulated question that uses explicit, systematic methods to identify, select, and critically appraise relevant research, and to collect and analyze data from the studies that are included in the review. These reviews differ substantially from narrative-based reviews or synthesis articles. Statistical methods (meta-analysis) may or may not be used to analyze and summarize the results of the included studies.

Reports of systematic reviews and meta-analyses should include a completed PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) checklist and flow diagram to accompany the main text. Blank templates are available here:

- Checklist: PDF or Word document
- Flow diagram: PDF or Word document

Authors must also state in their "Methods" section whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information and provide the registry number in the abstract.

If your article is a systematic review or a meta-analysis you should:

- State this in your cover letter
- Select "Research Article" as your article type when submitting
- Include the PRISMA flow diagram as Fig 1 (required where applicable)
- Include the PRISMA checklist as supporting information

Meta-analysis of genetic association studies

Manuscripts reporting a meta-analysis of genetic association studies must report results of value to the field and should be reported according to the guidelines presented in *Systematic Reviews of Genetic Association Studies* by Sagoo *et al.*

On submission, authors will be asked to justify the rationale for the meta-analysis and how it contributes to the base of scientific knowledge in the light of previously published results. Authors will also be asked to complete a checklist (DOCX) outlining information about the justification for the study and the methodology employed. Meta-analyses that replicate published studies will be rejected if the authors do not provide adequate justification.

Personal data from third-party sources

For all studies using personal data from internet-based and other third-party sources (e.g., social media, blogs, other internet sources, mobile phone companies), data must be collected and used according to company/website Terms and Conditions, with appropriate permissions. All data sources must be acknowledged clearly in the Materials and Methods section.

Read our policy on data availability.

In the Ethics Statement, authors should declare any potential risks to individuals or individual privacy, or affirm that in their assessment, the study posed no such risks. In addition, the following Ethics and Data Protection requirements must be met.

For interventional studies, which impact participants' experiences or data, the study design must have been prospectively approved by an Ethics Committee, and informed consent is required. The Ethics Committee may waive the requirement for approval and/or consent.

For observational studies in which personal experiences and accounts are not manipulated, consultation with an Ethics or Data Protection Committee is recommended. Additional requirements apply in the following circumstances:

- If information used could threaten personal privacy or damage the reputation of individuals whose data are used, an Ethics Committee should be consulted and informed consent obtained or specifically addressed.
- If authors accessed any personal identifying information, an Ethics or Data Protection Committee should oversee data anonymization. If data were anonymized and/or aggregated before access and analysis, informed consent is generally not required.

Note that Terms of Use contracts do not qualify as informed consent, even if they address the use of personal data for research.

See our reporting guidelines for human subjects research.

Cell lines

Authors reporting research using cell lines should state when and where they obtained the cells, giving the date and the name of the researcher, cell line repository, or commercial source (company) who provided the cells, as appropriate.

Authors must also include the following information for each cell line:

For *de novo* (new) cell lines, including those given to the researchers as a gift, authors must follow our policies for human subjects research or animal research, as appropriate. The ethics statement must include:

- Details of institutional review board or ethics committee approval; AND
- For human cells, confirmation of written informed consent from the donor, guardian, or next of kin

For established cell lines, the Methods section should include:

- A reference to the published article that first described the cell line; AND/OR
- The cell line repository or company the cell line was obtained from, the catalogue number, and whether the cell line was obtained directly from the repository/company or from another laboratory

Authors should check established cell lines using the ICLAC Database of Cross-contaminated or Misidentified Cell Lines to confirm they are not misidentified or contaminated. Cell line authentication is recommended – e.g., by karyotyping, isozyme analysis, or short tandem repeats (STR) analysis – and may be required during peer review or after publication.

Blots and gels

Please review *PLOS ONE's* requirements for reporting blot and gel results and providing the underlying raw images.

Antibodies

Manuscripts reporting experiments using antibodies should include the following information:

- The name of each antibody, a description of whether it is monoclonal or polyclonal, and the host species.
- The commercial supplier or source laboratory.
- The catalogue or clone number and, if known, the batch number.
- The antigen(s) used to raise the antibody.
- For established antibodies, a stable public identifier from the Antibody Registry.

The manuscript should also report the following experimental details:

- The final antibody concentration or dilution.
- A reference to the validation study if the antibody was previously validated. If not, provide details of how the authors validated the antibody for the applications and species used.

We encourage authors to consider adding information on new validations to a publicly available database such as Antibodypedia or CiteAb.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to initial submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a

recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Methods, software, databases, and tools

PLOS ONE will consider submissions that present new methods, software, databases, or tools as the primary focus of the manuscript if they meet the following criteria:

Utility
The tool must be of use to the community and must present a proven advantage over existing alternatives, where applicable. Recapitulation of existing methods, software, or databases is not useful and will not be considered for publication. Combining data and/or functionalities from other sources may be acceptable, but simpler instances (i.e. presenting a subset of an already existing database) may not be considered. For software, databases, and online tools, the long-term utility should also be discussed, as relevant. This discussion may include maintenance, the potential for future growth, and the stability of the hosting, as applicable.

Validation
Submissions presenting methods, software, databases, or tools must demonstrate that the new tool achieves its intended purpose. If similar options already exist, the submitted manuscript must demonstrate that the new tool is an improvement over existing options in some way. This requirement may be met by including a proof-of-principle experiment or analysis; if this is not possible, a discussion of the possible applications and some preliminary analysis may be sufficient.

Availability
If the manuscript's primary purpose is the description of new software or a new software package, this software must be open source, deposited in an appropriate archive, and conform to the Open Source Definition. If the manuscript mainly describes a database, this database must be open-access and hosted somewhere publicly accessible, and any software used to generate a database should also be open source. If relevant, databases should be open for appropriate deposition of additional data. Dependency on commercial software such as Mathematica and MATLAB does not preclude a paper from consideration, although complete open source solutions are preferred. In these cases, authors should provide a direct link to the deposited software or the database hosting site from within the paper. If the primary focus of a manuscript is the presentation of a new tool, such as a newly developed or modified questionnaire or scale, it should be openly available under a license no more restrictive than CC BY.

Software submissions

Manuscripts whose primary purpose is the description of new software must provide full details of the algorithms designed. Describe any dependencies on commercial products or operating system. Include details of the supplied test data and explain how to install and run the software. A brief description of enhancements made in the major releases of the software may also be given. Authors should provide a direct link to the deposited software from within the paper.

Database submissions

For descriptions of databases, provide details about how the data were curated, as well as plans for long-term database maintenance, growth, and stability. Authors should provide a direct link to the database hosting site from within the paper.

Read the PLOS policy on sharing materials, software and code.

New taxon names

Zoological names

When publishing papers that describe a new zoological taxon name, PLOS aims to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN). Effective 1 January 2012, the ICZN considers an online-only publication to be legitimate if it meets the criteria of archiving and is registered in ZooBank, the ICZN's official registry.

For proper registration of a new zoological taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> ***Anochetus boltoni*** *Fisher* ***sp. nov.*** *urn:lsid:zoobank.org:act:B6C072CF-1CA6-40C7-8396-534E91EF7FBB*

You will need to contact Zoobank to obtain a GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper.

Please also insert the following text into the **Methods** section, in a sub-section to be called “Nomenclatural Acts”:

> The electronic edition of this article conforms to the requirements of the amended International Code of Zoological Nomenclature, and hence the new names contained herein are available under that Code from the electronic edition of this article. This published work and the nomenclatural acts it contains have been registered in ZooBank, the online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub: XXXXXXX. The electronic edition of this work was published in a journal with an ISSN, and has been archived and is available from the following digital repositories: LOCKSS [author to insert any additional repositories].

All PLOS articles are deposited in LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Botanical names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature, and apply only to seed plants, ferns, and lycophytes.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> **Solanum aspersum** S.Knapp, sp. nov. [urn:lsid:ipni.org:names:77103633-1] Type: Colombia. Putumayo: vertiente oriental de la Cordillera, entre Sachamates y San Francisco de Sibundoy, 1600-1750 m, 30 Dec 1940, J. Cuatrecasas 11471 (holotype, COL; isotypes, F [F-1335119], US [US-1799731]).

Journal staff will contact IPNI to obtain the GUID (LSID) after your manuscript is accepted for publication, and this information will then be added to the manuscript during the production phase

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording:

> The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
>
> In addition, new names contained in this work have been submitted to IPNI, from where they will be made available to the Global Names Index. The IPNI LSIDs can be resolved and the associated information viewed through any standard web browser by appending the LSID contained in this publication to the prefix http://ipni.org/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (LOCKSS etc)].

All PLOS articles are deposited in LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Fungal names

When publishing papers that describe a new botanical taxon, PLOS aims to comply with the requirements of the International Code of Nomenclature for algae, fungi, and plants (ICN). The following guidelines for publication in an online-only journal have been agreed such that any scientific botanical name published by us is considered effectively published under the rules of the Code. Please note that these guidelines differ from those for zoological nomenclature.

Effective January 2012, the description or diagnosis of a new taxon can be in either Latin or English. This does not affect the requirements for scientific names, which are still to be Latin.

Also effective January 2012, the electronic PDF represents a published work according to the ICN for algae, fungi, and plants. Therefore the new names contained in the electronic publication of PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.

Additional information describing recent changes to the Code can be found here.

For proper registration of the new taxon, we require two specific statements to be included in your manuscript.

In the **Results** section, the globally unique identifier (GUID), currently in the form of a Life Science Identifier (LSID), should be listed under the new species name, for example:

> ***Hymenogaster huthii.*** Stielow et al. 2010, sp. nov. [urn:lsid:indexfungorum.org:names:518624]

You will need to contact either Mycobank or Index Fungorum to obtain the GUID (LSID). Please do this as early as possible to avoid delay of publication upon acceptance of your manuscript. It is your responsibility to provide us with this information so we can include it in the final published paper. Effective January 2013, all papers describing new fungal species must reference the identifier issued by a recognized repository in the protologue in order to be considered effectively published.

In the **Methods** section, include a sub-section called “Nomenclature” using the following wording. Note that this example is for taxon names submitted to MycoBank; please substitute appropriately if you have submitted to Index Fungorum using the prefix http://www.indexfungorum.org/Names/NamesRecord.asp?RecordID=.

> The electronic version of this article in Portable Document Format (PDF) in a work with an ISSN or ISBN will represent a published work according to the International Code of Nomenclature for algae, fungi, and plants, and hence the new names contained in the electronic publication of a PLOS article are effectively published under that Code from the electronic edition alone, so there is no longer any need to provide printed copies.
>
> In addition, new names contained in this work have been submitted to MycoBank from where they will be made available to the Global Names Index. The unique MycoBank number can be resolved and the associated information viewed through any standard web browser by appending the MycoBank number contained in this publication to the prefix http://www.mycobank.org/MB/. The online version of this work is archived and available from the following digital repositories: [INSERT NAMES OF DIGITAL REPOSITORIES WHERE ACCEPTED MANUSCRIPT WILL BE SUBMITTED (LOCKSS etc)].

All PLOS articles are deposited in LOCKSS. If your institute, or those of your co-authors, has its own repository, we recommend that you also deposit the published online article there and include the name in your article.

Qualitative research

Qualitative research studies use non-quantitative methods to address a defined research question that may not be accessible by quantitative methods, such as people's interpretations, experiences, and perspectives. The analysis methods are explicit, systematic, and reproducible, but the results do not involve numerical values or use statistics. Examples of qualitative data sources include, but are not limited to, interviews, text documents, audio/video recordings, and free-form answers to questionnaires and surveys.

Qualitative research studies should be reported in accordance to the Consolidated criteria for reporting qualitative research (COREQ) checklist or Standards for reporting qualitative research (SRQR) checklist. Further reporting guidelines can be found in the Equator Network's Guidelines for reporting qualitative research.

You may be eligible for APC support

Many institutional partners globally have publishing agreements with PLOS to allow their corresponding authors to publish with reduced or no APCs. To determine if your corresponding author is eligible, please visit our institutional partners page to determine what kind of agreement your institution has with PLOS.

If your corresponding author is affiliated with a participating institution, they must follow the instructions below to demonstrate eligibility.

Read the full instructions for submitting to a journal with the Flat Fee Agreement.

If your corresponding author is not from a participating institution and requires assistance paying publishing fees, please consider applying for a fee waiver at submission.