Validation of a Single-step, Single-tube Reverse Transcription-Loop-Mediated Isothermal Amplification Assay for Rapid Detection of SARS-CoV-2 RNA

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Abstract

In this study we undertook validation of a loop-mediated isothermal amplification (LAMP) assay using a commercial enzyme mastermix from OptiGene Ltd in combination with a primer set designed to detect the CDC N1 region of the SARS-CoV-2 nucleocapsid (N) gene. We implemented a single-tube, single-step fluorescence assay named N1-Single-Tube-Optigene LAMP (*N1-STOP-LAMP*) whereby as little as 1μL of universal transport medium (UTM) from a single nasopharyngeal swab could be used directly as template material, bypassing the requirement for RNA purification. Importantly, amplification and detection could be conducted in any thermocycler that could hold 65°C for 30 minutes and measure fluorescence in the FAM channel at one-minute intervals. Assay evaluation by assessment of 157 clinical specimens previously screened by E-gene RT-qPCR revealed assay sensitivity and specificity of 87% and 100%, respectively. Time-to-results were fast, with an average time-to-positive (Tp) for 93 clinical samples of 14 minutes (SD ±7 minutes). Using dilutions of SARS-CoV-2 virus spiked into UTM, we also evaluated assay performance against FDA guidelines for implementation of emergency-use diagnostics and established a limit-of-detection of 54 $TCID_{50}$/mL, with satisfactory assay sensitivity and specificity. A comparison of 20 clinical specimens between four laboratories showed excellent interlaboratory concordance, performing equally well on three different, commonly used thermocyclers across four laboratories, pointing to the robustness of the assay. With its simplified workflow, N1-STOP-LAMP is a powerful, scalable option for specific and rapid detection of SARS-CoV-2 and an additional resource in the diagnostic armamentarium against COVID-19.

48 ## Introduction

49 The current SARS-CoV-2 pandemic has created an unprecedented global demand for rapid
50 diagnostic testing. Most countries are employing real-time quantitative PCR (RT-qPCR) for
51 confirmation of infection (1-7). Consequently, a global shortage of RNA extraction kits as well as
52 RT-qPCR assay kits and their associated reagents has ensued (8-10). Thus, there is a need for
53 diagnostics that are not dependent on these commonly used materials. First described 20 years
54 ago by Notomi *et al.*, (2000), the loop-mediated isothermal amplification (LAMP) assay is robust,
55 rapid and straightforward, yet retains high sensitivity and specificity (11). These features have
56 seen the LAMP assay implemented for a broad range of molecular diagnostic applications
57 extending from infectious diseases, including detection of the original SARS-CoV-1 virus (12),
58 bacteria and parasites (13-15) to cancer (16). Using different reagents than RT-qPCR, with the
59 potential for direct processing of samples without the need for prior RNA extraction, improving on
60 an already rapid turn-around, several groups have now described different LAMP assays for
61 detection of SARS-CoV-2 RNA (17-24).

62

63 In this study, we developed a RT-LAMP assay that targets the CDC N1 region of the SARS-CoV-2
64 nucleocapsid gene (N-gene) (3) and used a commercial mastermix from OptiGene Ltd. This mix
65 contains a proprietary reverse transcriptase for cDNA synthesis and the thermophilic GspSSD
66 strand-displacing polymerase/reverse transcriptase for DNA amplification (www.optigene.co.uk)
67 with a dsDNA intercalating fluorescent dye. Detection was achieved by measuring the increase in
68 fluorescence as amplification products accumulate. The **N1** gene **S**ingle **T**ube **Op**tigene **LAMP**
69 assay (hereafter called N1-STOP-LAMP) was assessed for the direct detection of SARS-CoV-2 RNA,
70 following *FDA Policy for Diagnostic Tests for Coronavirus Disease-2019 during the Public Health*
71 *Emergency* against the four parameters and acceptance criteria summarized in Table 1 (5).

72 Validation samples were human upper respiratory tract specimens, collected using
73 nasopharyngeal flocked swabs, stored in universal transport media (UTM) or rayon dry swabs.

74

75 **Results**

76 *Assessing detection sensitivity of N1-STOP-LAMP using purified RNA*.

77 We began by assessing the limit of detection (LoD) of N1-STOP-LAMP under optimum conditions
78 using a 10-fold dilution series of purified RNA, prepared from a titred SARS-CoV-2 virus stock.
79 Although LAMP assays are not strictly quantitative, a positive correlation between time-to-positive
80 (Tp) and RNA concentration was evident. This was indicated by an absolute detection threshold
81 between 50 and 500 viral genome copies per reaction. We achieved a reliable detection of 5/5
82 replicates at 500 viral genome copies and detection of 4/5 replicates at 50 viral genome copies per
83 reaction (Fig. 1A). Of note, this threshold is likely an underestimate of the true detection limit, as
84 we assumed all RNA yielded from the viral stock generated from the Vero cell supernatant was of
85 viral origin. Using Tissue Culture Infectious Dose 50 ($TCID_{50}$), the absolute LoD of N1-STOP-LAMP
86 was between 0.001 and 0.01 $TCID_{50}$/reaction (equivalent to 1-10 $TCID_{50}$/mL).

87

88 *Direct detection of SARS-CoV-2 RNA in clinical specimens and specimen inactivation*

89 The nasopharyngeal specimens received by our public health laboratories were collected using
90 Copan flocked swabs in either 1mL or 3mL of universal transport media (UTM®). To assess the
91 impact of sample matrix on the LAMP assay, we performed pilot experiments with UTM from
92 swabs taken from SARS-CoV-2 negative specimens, adding increasing amounts of UTM to N1-
93 STOP-LAMP. We observed sterile UTM had no impact on N1-STOP-LAMP (data not shown), but
94 that 5µL of UTM with nasopharyngeal sample matrix used directly as the source of template RNA

95 was inhibitory to the assay (Fig 1B). This inhibition was relieved at a 1/5 dilution of UTM, thus 5µL
96 of a 1/5 UTM dilution or a 1µL neat UTM was selected as the optimum template volume for N1-
97 STOP-LAMP (Fig. 2A). Experiments were also conducted using dry swabs eluted in PBS. We tested
98 elution in 0.5mL, 1.0mL and 1.5mL and found that an elution volume of 1.5mL of PBS was a good
99 compromise between unnecessary dilution of potential virus in the sample and sufficient dilution
100 to alleviate assay inhibition (data not shown). To reduce the risk associated with handling clinical
101 specimens containing infectious SARS-CoV-2, we also assessed the impact of a heat inactivation
102 step on detection sensitivity. Pretreatment of UTM at 60°C for 30min led to a >5-$\log_{10}$ inactivation
103 of the virus accessed by PFU and $TCID_{50}$ determination (data not shown) and had no observable
104 impact on N1-STOP-LAMP detection sensitivity, with equivalent Tp between each treatment (Table
105 2, Fig. 1C).

106

107 *Comparative detection sensitivity of N1-STOP-LAMP versus E-gene RT-qPCR.*

108 The N1-STOP-LAMP assay was then directly compared with E-gene RT-qPCR, using a dilution of
109 titred SARS-CoV-2 virus stock in UTM across a 6-$\log_{10}$ dilution series. A RT-qPCR calibration curve
110 was established from triplicate extraction experiments for each of the six dilutions (Fig. 2A). The
111 assays were prepared with 5µL of purified viral RNA as template for both the N1-STOP-LAMP and
112 RT-qPCR assays. A 5µL and 1µL aliquot of each virus dilution in UTM was also tested directly in the
113 N1-STOP-LAMP assay (*i.e*. without RNA extraction). The side-by-side comparisons showed E-gene
114 RT-qPCR was up to 1-$\log_{10}$ more sensitive than N1-STOP-LAMP, with RT-qPCR detecting 2/3
115 replicates at the lowest dilution of 0.54 $TCID_{50}$/mL and N1-STOP-LAMP detecting no viral RNA at
116 this concentration (Table 3, Fig. 2). At 54 $TCID_{50}$/mL, the RT-qPCR and N1-STOP-LAMP detected 3/3
117 replicates. There was no difference in N1-STOP-LAMP sensitivity using either 5µL UTM added

118 directly to the test or purified RNA, but the assay lost another 1-log_{10} sensitivity where 1µL of neat
119 UTM was added directly to the N1-STOP-LAMP reaction (Fig. 2B).
120

121 *Establishing N1-STOP-LAMP limit of detection (LoD)*

122 The FDA guidelines for implementation of emergency-use diagnostics defines LoD as the lowest
123 concentration at which 19/20 replicates are positive (Table 1). Informed by the previous
124 experiment (Fig. 2B), we selected a SARS-CoV-2 concentration of 54 $TCID_{50}$/mL (in UTM) and
125 tested 20x 1µL aliquots by N1-STOP-LAMP. We observed 20/20 positive reactions with an average
126 Tp of 15.8 mins, inter-quartile range 12-19 mins and coefficient of variation of 31.18% (Fig. 3A).
127

128 *Evaluation of N1-STOP-LAMP against FDA criteria.*

129 The FDA guidelines for implementation of emergency-use diagnostics also require an assessment
130 of assay performance using at least 30 contrived positive and 30 negative clinical specimens
131 (authentic or contrived), with at least 20 of the positives at a concentration of 1-2x the LoD (Table
132 1). We obtained 30 nasopharyngeal dry swabs from healthy, anonymous volunteers. Swabs were
133 eluted in 1.5mL of PBS and 1µL aliquots were tested directly by N1-STOP-LAMP. All eluates were
134 negative. We then spiked the eluates with SARS-CoV-2 virus at 1x and 2x the LoD (approx. 50 and
135 100 $TCID_{50}$/mL respectively). Results showed that N1-STOP-LAMP detected 20/20 samples spiked
136 with 1x the assay LoD and 10/10 samples at 2x LoD (Fig. 3B, Table 4). N1-STOP-LAMP thus meets
137 the FDA *Clinical Evaluation* criteria (Table 1).
138

139 *Clinical specimen evaluation of N1-STOP-LAMP against E-gene RT-qPCR Gold Standard*

140 We then sought to evaluate N1-STOP-LAMP performance against E-gene RT-qPCR, setting the
141 latter assay as the current 'gold standard'. We screened 50 negative clinical specimens and 107
142 positive nasopharyngeal clinical specimens in UTM as established by E-gene RT-qPCR (Table S1).
143 Results showed N1-STOP-LAMP assay sensitivity of 87%, specificity of 100%, positive predictive
144 value of 100% and negative predictive value of 78% (Table 5, Fig. 3). As noted during initial
145 sensitivity experiments (Table 3), a positive correlation was observed between N1-STOP-LAMP Tp
146 and E-gene RT-qPCR Ct (Fig. 4). The average Tp among the 93 N1-LAMP-STOP positive specimens
147 was 14 min (SD $\pm$ 7 min). For the false-negative samples with sufficient clinical material remaining
148 (5 out of 14), RNA extraction using a simple, rapid magnetic bead purification protocol was
149 performed, then N1-STOP-LAMP repeated. Four of the 5 samples returned a positive result with
150 Tp range from 10:00 – 25:30 min.sec) (Table S1). Indicating that the reduced sensitivity of N1-
151 STOP-LAMP compared to RT-qPCR can be improved upon by using samples with a higher RNA
152 template concentration and purity with only a small increase in time to results.

153

154 *Confirmation of N1-STOP-LAMP inclusivity and exclusivity*

155 The FDA guidelines for implementation of emergency-use diagnostics also require an assessment
156 of inclusivity and exclusivity of the oligonucleotide primers used in an assay. To assess the former,
157 we performed *in silico* screening of the region spanned by the six primers used for the N1-STOP-
158 LAMP assay against all available SARS-CoV-2 genome sequences (Supplementary data file 1). This
159 240bp sequence spans the CDC N1 region of the SARS-CoV-2 N-gene (3, 25). Alignment of this
160 region against 2738 genomes revealed 100% conservation amongst all entries, showing that this a
161 conserved target sequence and that the primers used will perform equally across all lineages of
162 the virus.

163 To assess the exclusivity criterion, we used a nucleotide BLAST search of the N1 region against the
164 NCBI Genbank *nt* database and observed no non-SARS-CoV-2 sequence matches above 80%
165 nucleotide identity, in-line with FDA cross-reactivity requirement. Further to this analysis, we also
166 performed *in vitro* testing with the NATRPC2-BIO (ZeptoMetrix) specificity panel, a control panel
167 consisting of 22 common respiratory pathogens. None of these pathogens were detected by N1-
168 STOP-LAMP (Table S3). Thus, both *in silico* and *in vitro* testing confirmed the specificity of N1-
169 STOP-LAMP for SARS-CoV-2.

170

171 *Interlaboratory comparisons*

172 In order to explore the ability of other laboratories to readily deploy N1-STOP-LAMP, we prepared
173 an external-quality-assessment (EQA) panel of 20 clinical specimens (Table S2). These were
174 previous positive and negative clinical samples as assessed by E-gene RT-qPCR that had been heat-
175 inactivated for ease-of-handling. These specimens covered a range of virus titres (Ct values 16.3 –
176 30.6). The panel was tested simultaneously in four different laboratories (named Labs A – D). The
177 results showed excellent correspondence between all laboratories for the nine positive samples
178 with E-gene RT-qPCR Ct values <26 (Fig. 5, Table S2). Above this Ct (*i.e.* those samples with lower
179 virus titres), the laboratories returned variably positive results (Samples 14, 16, 17 & 18), reflecting
180 that these specimens had virus concentrations at or beyond the N1-STOP-LAMP LoD (Table S2).
181 Concordance among the seven negative results was overall very good, however one laboratory
182 (Lab C) returned a false positive result (Sample 2), highlighting the issue of contamination with
183 sensitive molecular tests (Table S2). Of note, the laboratories used a range of different
184 instruments for amplification/detection including an OptiGene Genie II, BioRad CFX and
185 ThermoFisher Quantstudio-7. This trial suggests that N1-STOP-LAMP is a robust and transferrable
186 assay format.

187

188 ## Discussion

189 A critical component of an effective COVID-19 pandemic response is the rapid and robust
190 detection of positive clinical samples (26). This has required massively upscaled SARS-CoV-2
191 diagnostic capabilities. and in particularl, a worldwide focus on developing improved technologies
192 (27). There have been more than 20 molecular tests recently receiving FDA Emergency Use
193 Authorizations (EUAs) (www.fda.gov/medical-devices/emergency-situations-medical-
194 devices/emergency-use-authorizations#covid19ivd, accessed 11/04/2020). In this current study,
195 we demonstrate the potential of N1-STOP-LAMP. We focused efforts on the LAMP assay because
196 it has been previously employed for the rapid and robust detection of numerous RNA viruses
197 including Zika, Chikungunya, Influenza and SARS-CoV-1 (28-30) and was of great assistance during
198 these outbreaks, particularly in resource constrained settings. Advantages of LAMP testing include
199 rapid turnaround time, ease of implementation, non-standard reagent use and potential utility at
200 point of care (19-21). To date, there has been limited detail on the performance characteristics of
201 emerging LAMP-based tests in head-to-head comparisons with gold standard assays. Such
202 information is critical to ensuring the safe deployment of new tests, given the clinical and public
203 health consequences of erroneous test results.

204

205 Here we have shown the specific detection of SARS-CoV-2 RNA directly from clinical swabs without
206 the need for RNA purification, using a LAMP assay targeting the N1 region of the SARS-CoV-2 N-
207 gene in conjunction with the reverse transcriptase/GspSSD LF DNA polymerase mastermix from
208 OptiGene Ltd. With a detection limit of 54 $TCID_{50}$/mL the N1-STOP-LAMP assay met each of the
209 four FDA criteria for EUA. Based on reported data, as opposed to head-to-head comparisons, the
210 N1-STOP-LAMP detection limit, is higher than several recently reported FDA-EUA COVID-19 tests.

211 Expressed as virus copies/mL the N1-STOP-LAMP LoD is estimated at 54,000 virus copies/mL
212 (assumes our $TCID_{50}$ underestimates virus copy number by 1000-fold, see methods), compared to
213 Cepheid GeneXpert Xpress 250 virus copies/mL or Abbott ID NOW COVID-19 test 125 virus
214 copies/mL. Other EUA assays reporting LoDs similar to N1-STOP-LAMP include the Luminex ARIES
215 SARS-CoV-2 assay (75,000 copies/mL) and the GenMark ePlex SARS-CoV-2 test (100,000
216 copies/mL). Given these performance metrics, and the test's high positive predictive value (100%),
217 we see N1-STOP-LAMP as well suited for widespread screening of large populations when COVID-
218 19 prevalence is low. N1-STOP-LAMP could be used in large-scale, national testing programs to
219 support aggressive COVID-19 contact tracing and disease suppression or elimination activities. The
220 test could also play a role in near-point-of-care settings, such as outbreak investigation in hospitals
221 and nursing homes, providing rapid-turnaround-time of results for health-care workers and highly
222 vulnerable patients. The relatively simple assay format of N1-STOP-LAMP is also suitable for
223 deployment in lower and middle-income countries, where access to sophisticated laboratory
224 infrastructure is limited.

225

226 During this evaluation we noted opportunities for improvement of N1-STOP-LAMP. We observed
227 that although 1µL of UTM was tolerated in the OptiGene RT-LAMP formulation, larger quantities
228 of sample matrix (UTM or PBS containing additional human material from the nasopharyngeal
229 swab) were inhibitory to the RT-LAMP reaction. If more than 1µL of sample matrix was used in the
230 assay, no amplification was observed using the purified virus or RNA as template spiked into UTM
231 or PBS (Fig. 1B). However, under ideal conditions (*i.e.*, no sample inhibition from human
232 components), the impact of using an increased template volume was shown, where 5µL of UTM
233 added directly to the N1-STOP-LAMP reaction was equal to the sensitivity of RT-qPCR using
234 purified RNA (Fig. 2). Thus, the addition of a simple step to purify RNA improves the detection

235 sensitivity of N1-STOP-LAMP to rival RT-qPCR. Our follow-up of five false-negative results (Table 5,
236 Fig. 3C), demonstrated that inclusion of such a step in the assay is feasible. We used a simple,
237 scalable and rapid magnetic-bead RNA purification step and we were able to detect SARS-CoV-2
238 RNA with N1-STOP-LAMP in 4/5 UTM specimens that had previously tested negative by the assay
239 (Table S1) (31). Other opportunities for improvement include the use of specific fluorescent
240 probes to detect amplification products, thus facilitating multiplex reactions and the inclusion of
241 an internal amplification control within each test (24, 32).

242

243 As mentioned, recognised strengths of the LAMP assay include the rapid time to test result, with
244 the majority of the positive clinical samples yielding a result in under 15 min, and the robust test
245 format, testing directly from the swab eluate. Our preferred specimen type for N1-STOP-LAMP is a
246 dry nasopharyngeal swab. Detailed examination of optimum swabs for the detection of influenza
247 and other RNA viruses has shown dry swabs offer superior performance (33). In the current study
248 we have shown that N1-STOP-LAMP has satisfactory performance using dry swabs eluted in 1.5mL
249 of phosphate-buffered saline (Fig. 3B); a format that potentially doubles the effective
250 concentration of virus in the sample compared to 3mL of UTM in the Copan format. Another
251 strength of N1-STOP-LAMP is that the assay readily scales from small sample numbers (e.g. 8-well
252 with portable detection unit) to high throughput (run with sample robotics and 96-well
253 thermocyclers in a centralized laboratory).

254

255 While establishing the assay, we noted susceptibility to contamination that was easily addressed
256 by the standard precautions used to prevent template carryover for any nucleic acid amplification
257 test, such as: physical separation of mastermix preparation from sample inoculation; not opening
258 post-amplification reaction tubes or plates; and inclusion of negative extraction controls. Addition

259 of uracil-N-glycosylase has been reported as a strategy to limit the risk of amplicon contamination,
260 however this is associated with a loss of detection sensitivity (34).

261

262 In this report we have shown N1-STOP-LAMP is robust diagnostic test for the specific and rapid
263 detection of SARS-CoV-2. It is an alternative molecular test for SARS-CoV-2 that can be readily and
264 seamlessly deployed, particularly when access to standard RT-qPCR-based approaches are limited.

265

266 Materials and methods:

267 *Specimen collection and handling*

268 Nasopharyngeal swabs were collected by qualified healthcare professionals from patients meeting
269 the epidemiological and clinical criteria as specified by the Victorian Department of Health and
270 Human Services (DHHS) at the time of swab collection (1) between the 23rd of March 2020 and 4th
271 of April 2020. Copan flocked swabs with either 1mL or 3mL of universal transport medium (UTM®)
272 (Catalogue Nos., 330C and 350C respectively) were used. Samples were collected at metropolitan
273 hospitals in Melbourne, Victoria, Australia, and transported as per World Health Organization
274 recommendations (6). All swabs were processed in a class II biological safety cabinet.

275

276 *Cell culture and SARS-CoV-2 RNA extraction*

277 Vero cells (within 30 passages from the original American Type Culture Collection [ATCC] stock)
278 were maintained in Minimal Essential Media (MEM) supplemented with 10% heat-inactivated fetal
279 bovine serum (FBS), 10µM HEPES, 2mM glutamine and antibiotics. Cell cultures were maintained
280 at 37°C in a 5% CO_2 incubator. All virus infection cultures were conducted within the High
281 Containment Facilities in a PC3 laboratory at the Doherty Institute. To generate stocks of SARS-

282 CoV-2, confluent Vero cell monolayers were washed once with MEM without FBS (infection
283 media) then infected with a known amount SARS-CoV-2 virus originally isolated from a patient
284 (35). After 1h incubation at 37°C in a 5% CO2 incubator to enable virus binding, infection media
285 containing 1 mg/mL TPCK-trypsin was added and flasks returned to the incubator. After 3d
286 incubation and microscopic confirmation of widespread cytopathic effect (CPE), the supernatants
287 were harvested, and filter sterilized through a 0.45µm syringe filter. To assess infectious SARS-
288 CoV-2 viral titres, both $TCID_{50}$ and plaque assays were performed. Briefly, serial dilutions of the
289 stock virus were added to washed monolayers of Vero cells. After 1h incubation to allow virus to
290 adhere, for the $TCID_{50}$ assay, infection media containing 1µg/mL TPCK-Trypsin was added, while
291 for the plaque assay the infected cell monolayer was overlaid with Leibovitz-15 (L15) media
292 supplemented with 0.9% agarose (DNA grade, Sigma), antibiotics and 2µg/mL TPCK Trypsin. After
293 3d incubation, the dilution of stock required to cause CPE in at least 50% of wells ($TCID_{50}$) was
294 determined via back calculation of microscopic confirmation of CPE in wells for a given dilution.
295 For quantitation via plaque assay, plaques present in the monolayer were macroscopically
296 visualized, individually counted and back calculated for each given dilution to determine the
297 number of Plaque Forming Units (PFU) per mL in the original stock. Stocks of SARS-CoV-2 used by
298 this study had a $TCID_{50}$ of $5.4x10^5$ per mL and plaque assay gave $9.78x10^5$ PFU/mL. To heat
299 inactivate the virus, 200µL of neat stock was heated to 60°C for 30min, then cooled. Inactivation
300 was confirmed via complete lack of CPE and plaque formation using both $TCID_{50}$ and plaque
301 assays. To prepare SARS-CoV-2 RNA from stocks, 500µL aliquots were thawed and RNA extracted
302 using the RNEasy mini Kit (Qiagen) according to the manufacturer's specifications, with an elution
303 volume of 50µL. Based on RNA concentrations, the total virus harvested from Vero cell culture was
304 $3.1x10^9$ copies/mL, suggesting a high number of non-infectious virus particles in the virus stocks.

305

306 *RNA extraction from UTM for RT-qPCR*

307 A 200µL aliquot of Copan UTM® was processed through the QIAsymphony DSP Virus/Pathogen
308 Mini Kit (Qiagen, Cat# 937036) following manufactures instructions on the QIAsymphony SP
309 instrument. The RNA was eluted in 60µL of recommended buffer.

310

311 *RNA extraction from UTM SPRI beads for N1-STOP-LAMP*

312 A 50µL aliquot of solid-phase reversible immobilization (SPRI) on carboxylated paramagnetic beads
313 (Sera-Mag™ Magnetic SpeedBeads™, from GE Healthcare) were prepared for RNA binding as
314 described (https://openwetware.org/wiki/SPRI_bead_mix). RNA purification was performed in 96-
315 well plates with initial lysis by the addition of 25µL of 6GTD lysis buffer (7.08g/10mL guanidine
316 thiocyanate, made up to 8.2mL with 1mL Tris HCL [pH 8.0] and 800 µL of 1M dithiothreitol), mixed
317 by pipetting ten times and incubation at room temperature for 1 min (31). To this, 75µL of 100%
318 ethyl alcohol and 20µL of prepared SPRI beads were added, mixed by pipetting ten times and
319 incubated at room temperature for 5 min. The RNA-bead complex was then immobilized by
320 placing the 96-well plate on a magnetic rack and incubated again at room temperature for 5 min.
321 The supernatant was then discarded, and the beads washed twice in 200µL of freshly prepared
322 80% ethyl alcohol (v/v) with 30 sec room temperature incubation between each wash. The beads
323 were air-dried for 2 min at room temperature before RNA was eluted by the addition of 20µL of
324 nuclease-free water.

325

326 *E-gene RT-qPCR*

327 A one step RT-qPCR was conducted with the primers as described by Corman *et al*., targeting the
328 viral envelope E-gene of SARS-CoV-2 E_Sarbeco_F: 5'-ACAGGTACGTTAATAGTTAATAGCGT-3', 5'-

329 E_Sarbeco_R: ATATTGCAGCAGTACGCACACA-3', E_Sarbeco_P: 5'-FAM-
330 ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3') (36). A 20µL reaction was assembled consisting of 1x
331 qScript XLT One-Step RT-qPCR ToughMix Low ROX (2x) (QuantaBio), 400nM E_Sarbeco_F, 400nM
332 E_Sarbeco_R 200nM E_Sarbeco_P (Probe) and 5µL of purified RNA. The following program was
333 conducted on an ABI 7500 Fast instrument: 55°C for 10 min for reverse transcription, 1 cycle of
334 95°C for 3 min and then 45 cycles of 95°C for 15 sec and 58°C for 30 sec.

335

336 *N1-STOP-LAMP*

337 A 50-reaction bottle of dried Reverse Transcriptase isothermal mastermix (Optigene, ISO-DR004-
338 RT) was rehydrated with 750µL of resuspension buffer and vortexed gently to mix. Unused
339 mastermix was stored at -20°C. The mix contains both a proprietary reverse transcriptase and the
340 GspSSD LF DNA polymerase in the mastermix that has both reverse transcriptase and strand-
341 displacing DNA polymerase activity. The N1-STOP-LAMP assay uses six standard LAMP
342 oligonucleotide primers that target the sequence spanning the CDC N1 region of the SARS-CoV-2
343 nucleocapsid gene. The sequences of the primers are available upon request (GeneWorks Ltd).
344 Each N1-STOP-LAMP reaction contained, 15µL of mastermix, 5µL of 5x primer stock and 4µL of
345 water. Mastermix was reconstituted in a separate biological safety cabinet to that used for
346 template addition. The source of RNA template for the reaction consisted of either 1 µL of purified
347 SARS-CoV-2 RNA, 1 µL of SARS-CoV-2 purified virus or 1 µL of UTM from a nasopharyngeal swab. A
348 no-template control (1 µL water) was included in all runs. Reactions were assembled in either 8-
349 tube Genie strips (OP-00008, OptiGene Ltd) or 96-MicroAmp-Fast-Optical reaction plate (Applied
350 Biosystems). Reactions were capped or, for 96-MicroAmp-Fast-Optical reaction plates, sealed with
351 MicroAmp Optical adhesive film (Applied Biosystems) and tapped to remove bubbles. Strip tubes
352 were loaded onto the Genie-II or Genie-III (OptiGene Ltd) and 96-well plates run on a QuantStudio

353 7 thermocycler (ThermoFisher). Reactions were incubated at 65°C for 30 min with fluorescence
354 acquisition every 30 sec (Genie instruments) or 1 min (QuantStudio 7). A positive result was
355 indicated by an increase in fluorescence at an emission wavelength of 540nm (FAM channel)
356 above a defined threshold, recorded as time-to-positive (Tp, expressed in min.sec).

357

358 *Dilution series of purified SARS-CoV-2*

359 A virus stock with of 5.4 x10^5 $TCID_{50}$/mL was serially diluted to 10^{-6} in a generic universal transport
360 medium (comprising per liter: 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4, 0.2g KH_2PO_4, 4mL 0.5% phenol
361 red 0.5%, 5g gelatin, 950mL tissue culture water, 1mL Fungizone [5mg/mL], 20mL
362 penicillin/streptomycin) in biological triplicates with the 10^{-1} through to the 10^{-6} dilutions tested
363 by N1-STOP-LAMP (1μL). RNA was extracted from a 200μL aliquot of each dilution as described
364 above and 5μL of the purified RNA used as template in E-gene qRT-PCR or N1-STOP-LAMP assay.

365

366 *Interlaboratory comparison of N1-STOP-LAMP*

367 A panel of 20 blinded clinical samples (13 positive and 7 negative) with Ct values previously
368 established by E-gene RT-qPCR were aliquoted and distributed to three different laboratories for
369 independent testing by N1-STOP-LAMP assay (Table S2).

370

371 *Biological specificity*

372 To test for cross reactivity of the N1-STOP-LAMP assay, a control panel of respiratory pathogens
373 (NATRPC2-BIO, ZeptoMetrix) was screened (Table S3. A 1 μL aliquot of NATrol RP1 or RP2, or
374 samples spiked with SARS-CoV-2 virus were used as template for N1-STOP-LAMP.

375

376 *In silico nucleotide sequence comparisons of the N1 region of SARS-CoV-2*

377 To assess the inclusivity and exclusivity of the N1 region targeted by the LAMP assay 2755 publicly
378 available SARS-CoV-2 genomes were downloaded and filtered to remove entries of less than 29kb
379 using *Seqtk seq* (v1.3-r106). Sequence gaps were replaced with 'N' using *Seqkit* (v0.12.0). After this
380 quality control step, homology searches were then conducted using *NCBI BLAST+ blastn* using
381 *Genepuller.pl* (https://github.com/tseemann/bioinfo-scripts/blob/master/bin/gene-puller.pl) to
382 find the region in each of the remaining 2738 genomes matching the 5' region of the N1 sequence.
383 The resulting sequence coordinates of those hits were then used to extract the 230bp region from
384 all 2738 genomes using *Genepuller.pl* and aligned with Clustalo (v1.2.4). The alignment was
385 visualized with *Mesquite* (v3.61).

386

387 *Statistical analysis*

388 Data analysis was managed using GraphPad Prism (v8.4.1). Sensitivity and specificity testing were
389 performed using the Wilson/Brown hybrid method as deployed in GraphPad Prism.

390

391 *Ethics statement*

392 This study was conducted in accordance with the National Health and Medical Research Council of
393 Australia National Statement for Ethical Conduct in Human Research 2007 (Updated 2018). The
394 study was exempt from requiring specific approvals, as it involved the use of existing collections of
395 data or records that contained non-identifiable data about human beings (37).

396

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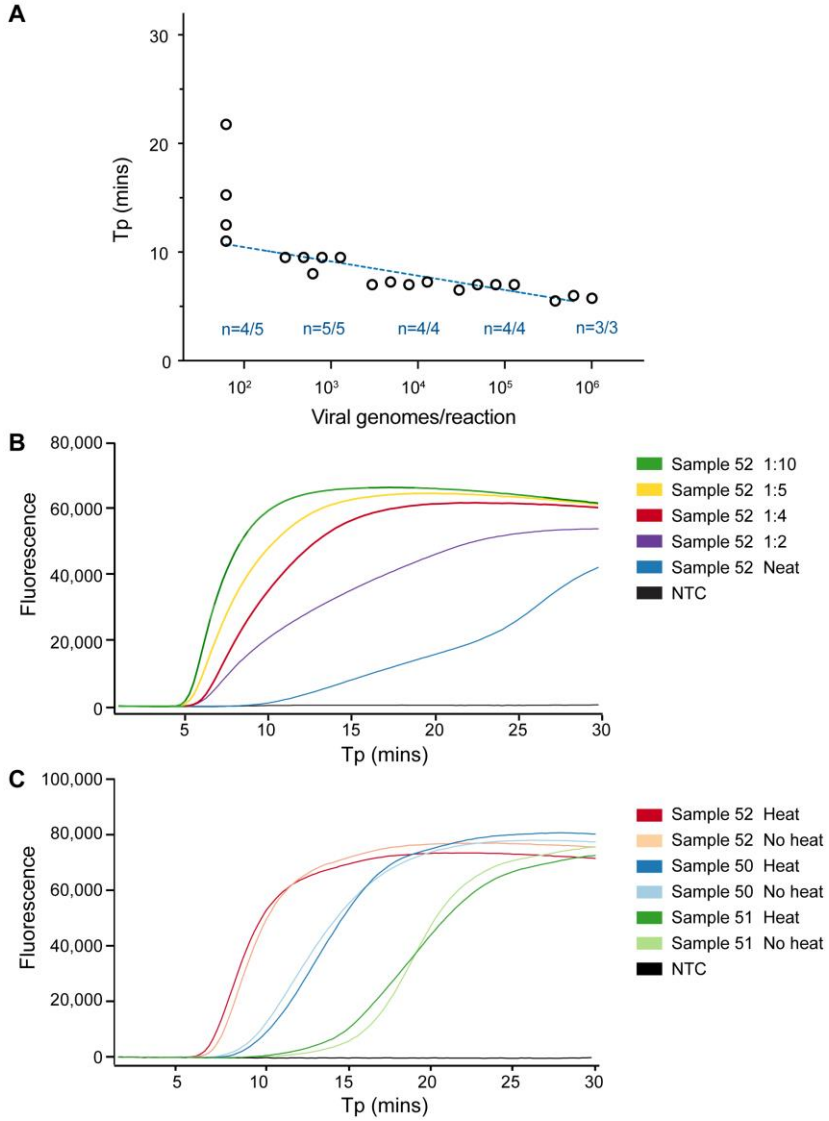
530 **Figures**



531 Fig. 1

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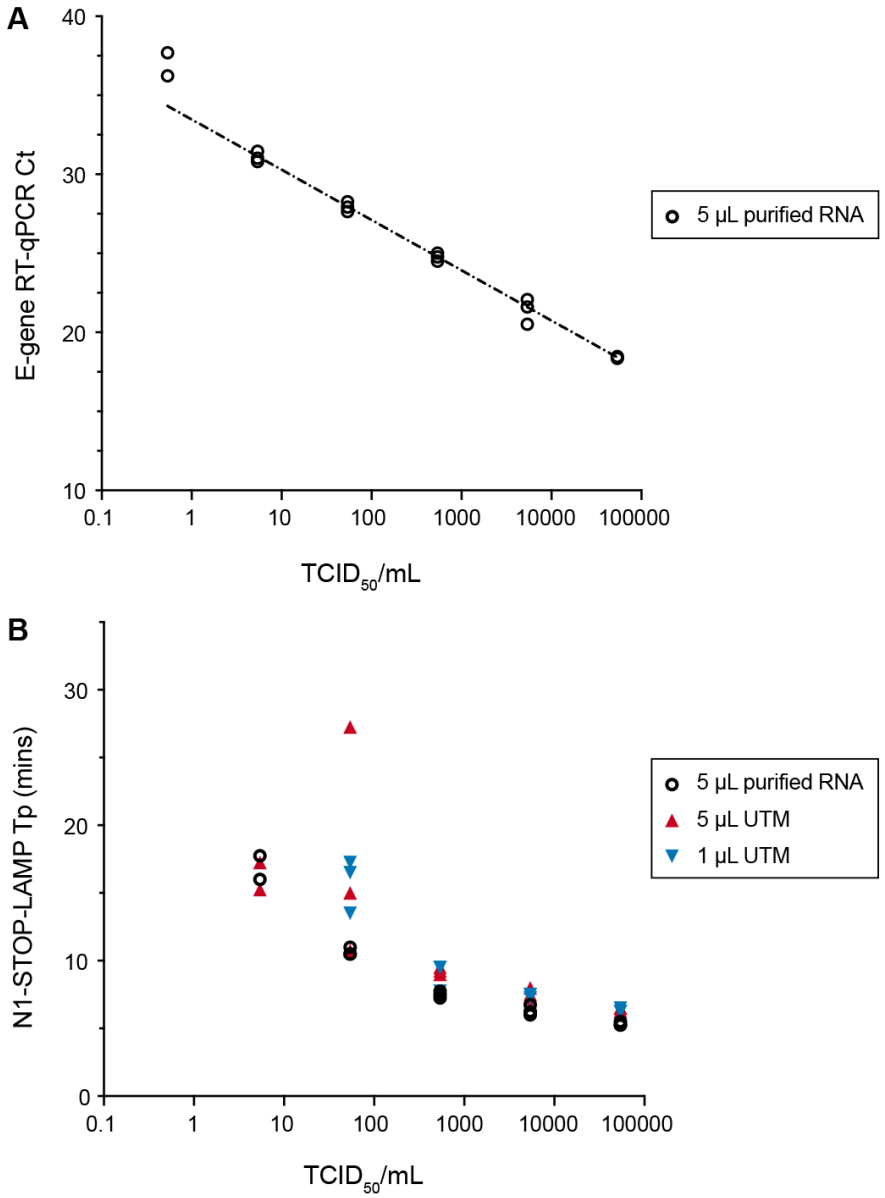
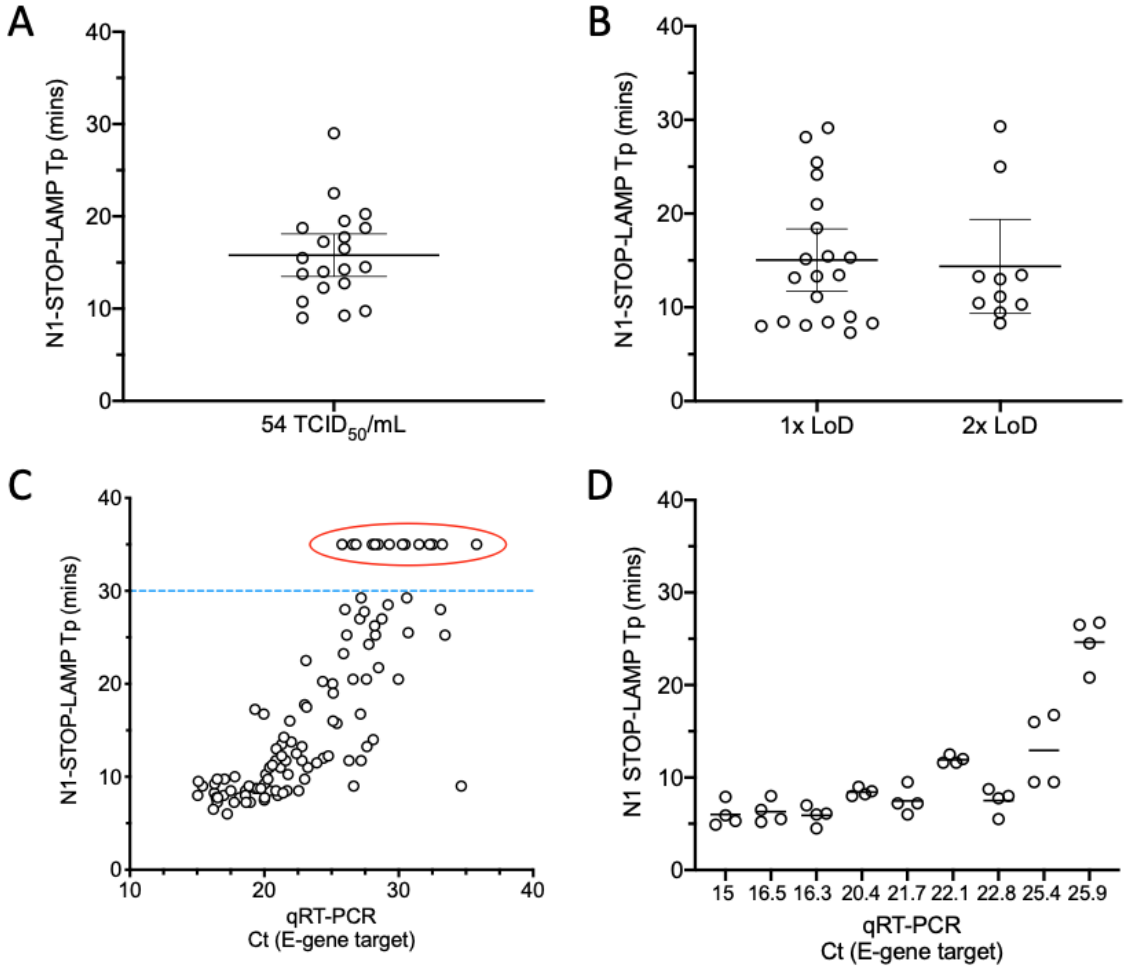
Fig. 1: *Limit of detection of N1 STOP-LAMP and establishing optimal sample template volume and inactivation conditions. (A) Plot showing performance of the N1 LAMP assay across 5-log$_{10}$ dilution of purified viral RNA. Y-axis is time-to-positive (Tp) and x-axis is estimate of viral genomes/reaction based on starting RNA concentration. The number of replicates per dilution (n) and number of positive replicates per dilution is indicated. (B) Example N1-STOP-LAMP amplification plots for SARS-CoV-2 positive clinical sample No. 52, showing inhibitory impact of 5uL of a neat sample matrix on LAMP and the effect of diluting the UTM in water. (C) N1-STOP-LAMP amplification plots for three SARS-CoV-2 positive clinical samples, showing no loss in detection sensitivity after specimen heat-treatment of 60°C for 30min to inactivate virus.*



533 **Fig. 2**

534

Fig. 2: *Comparison of E-gene RT-qPCR with N1-STOP-LAMP and limit of detection. Titred virus was serially diluted 10-fold in Universal Transport Medium (UTM) in triplicate and RNA was extracted from each replicate dilution. A 1µL aliquot of each UTM dilution was also tested directly by N1-STOP-LAMP. (A) Shows calibration curve for the E-gene RT-qPCR. A curve was interpolated using linear regression. (B) Comparative performance of N1-STOP-LAMP with 5µL purified RNA versus 1µL or 5µL of UTM directly added to the reaction.*



535 **Fig. 3**

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Fig. 3: *Clinical evaluation of N1-STOP-LAMP. (A) Establishing limit of detection according to FDA Emergency Use Authorizations (EUA) guidelines. Twenty, 1µL replicates of SARS-CoV-2 virus in Universal Transport Medium (UTM) at a concentration of 54 $TCID_{50}$/mL were tested by direct N1-STOP-LAMP. All data points plotted, with average and 95% CI indicated. (B) Assessment of assay against FDA EUA clinical performance criteria. Thirty healthy volunteer dry nasopharyngeal swabs eluted in 1.5mL of PBS were screened by N1-STOP-LAMP. Shown are time-to-positives (Tp). Mean and 95% CI are indicated. (C). Plot showing correspondence between 107 E-gene RT-qPCR positive and N1-STOP-LAMP, where 1uL of clinical specimen in UTM was added directly to the RT-LAMP reaction. Y-axis is LAMP Tp and x-axis is RT-qPCR cycle threshold (Ct). The dotted line indicates Tp 30 mins. Results plotted above this line (encircled) were RT-qPCR positive but N1-STOP-LAMP negative. (D) Results of 4-way interlaboratory comparison of N1-STOP-LAMP. Nine clinical samples previously tested positive by E-gene RT-qPCR were used to create a test panel for comparing assay performance between laboratories.*

538 ## Tables

539 **Table 1:** FDA validation study recommendations for SARS-CoV-2 molecular diagnostics (5)

Method Validation Parameter	Validation description and acceptance criteria
1. Limit of Detection (LoD)	Test a dilution series of three replicates per concentration. LoD defined as lowest concentration at which 19/20 replicates positive.
2. Clinical evaluation	Test at least 30 contrived reactive specimens and 30 non-reactive specimens. At least 20 specimens should have virus/RNA concentration of 1-2x LoD, remainder spanning the assay range. Acceptance defined as 95% agreement 1-2x LoD, and 100% agreement at other concentrations and for negative specimens.
3. Inclusivity	*In silico* analysis indicating percent identity matches of target region against publicly available SARS-CoV-2 sequences. Acceptance defined as 100% match against all entries with the selected primers/probes.
4. Cross-reactivity	*In silico* analysis of the assay primers/probes compared to respiratory microbiota and other viral pathogens. Acceptance defined as <80% homology between all primers/probes and non-target sequences present in public databases.

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542 **Table 2:** Assessment of heat treatment of UTM specimens on detection sensitivity

		Treatment	
Sample No	**E-gene RT-qPCR Ct**	**No heat (Tp, min:sec)**	**Heat (Tp, min:sec)**
50	21.76	10:15	10:45
51	24.40	17:45	15:45
52	15.05	7:15	7:45

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552 **Table 3:** Comparison of N1-STOP-LAMP and RT-qPCR performance using titred virus diluted in

		N1-LAMP (1uL direct)		N1-LAMP (5uL direct)		N1-LAMP (5uL of 60uL)		RT-qPCR (5uL of 60uL)	
SARS-CoV-2 ($TCID_{50}$/mL)	N	Tp[a] (Avg)	CV[b] (%)	Tp (Avg)	CV (%)	Tp (Avg)	CV (%)	Ct (Avg)	CV (%)
54000	3	6.33	2.28	6.41	2.25	5.33	2.71	18.41	0.33
5400	3	7.42	1.95	7.58	5.04	6.33	6.03	21.40	3.75
540	3	8.92	11.33	9.25	2.70	7.50	3.33	24.77	1.03
54	3	15.75	12.60	17.67	48.49	10.67	2.71	27.94	1.13
5.4	3	nd[c]	-	16.25	8.70[d]	16.88	7.33[d]	31.10	1.17
0.54	3	nd	-	nd	nd	nd	-	36.96	2.79[d]

553 UTM

554 **Notes:** [a]Tp= time-to-positive; [b]CV=Coefficient of Variation; [c]nd=not detected; [d]2/3 replicates positive at this
555 dilution

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558 **Table 4:** Summary of clinical evaluation comparisons against FDA criteria

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	Negative	1-2x LoD[a]	2x LoD
N1-STOP-LAMP +	0	20 (7:30 – 22:00)	10 (8:30 – 21:00)
N1-STOP-LAMP -	30	0	0

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564 **Notes:** [a]Parentheses indicate Tp range in min.sec

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568 **Table 5:** Comparison of N1-STOP-LAMP versus E-gene RT-qPCR using clinical specimens

	RT-qPCR +	RT-qPCR -	Total
N1-STOP-LAMP +	93	0	93
N1-STOP-LAMP -	14	50	64
Total	107	50	157

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570 ## Supplementary data

571

572 **Table S1**: Clinical data positivity summary (107 E-gene RT-qPCR positives vs N1-STOP-LAMP Tp)

Sample	E-gene RT-qPCR Ct	N1-STOP-LAMP Tp (min.sec)	N1-STOP-LAMP retest of RNA
1	23	17:45	
2	21	8:05	
3	23	9:45	
4	26	28:00	
5	nd	nd	
6	21.198	11:00	
7	17.803	10:00	
8	16.286	8:15	
9	19.312	8:45	
10	18.613	8:30	
11	25.08	20:00	
12	22	13:45	
13	28.1	14:00	
14	23.24	11:00	
15	24.34	20:15	
16	28.27	29:15	
17	23.14	17:30	
18	26.13	28:15	
19	16.43	7:45	
20	20.86	11:45	
21	27.15	16:45	
22	20.11	10:15	
23	20.4	11:00	
24	20	8:45	
25	18.86	9:00	
26	21.6	11:45	
27	27.1	27:00	
28	21.9	16:00	
29	17.04	9:45	
30	15.4	9:00	
31	19.3	17:15	
32	20.6	11:15	
33	15.1	9:30	
34	27.6	20:30	
35	28.5	21:45	
36	16.3	9:15	
37	16.5	9:45	
38	23.1	22:30	

39	31.51	nd		
40	30.46	nd		
41	25.9	23:15		
42	21.3	13:30		
43	30.6	29:15		
44	25.4	15:45		
45	22.8	13:15		
46	27.2	29:15		
47	21.7	8:30		
48	16.5	7:15		
49	20.4	8:30		
50	21.76	10.15		
51	24.4	12:00		
52	15.05	8		
53	28.75	27		
54	18.17	7.45		
55	17.24	6		
56	26.65	9		
57	23.9	11.3		
58	16.21	6.3		
59	17.76	7.15		
60	27.78	24.15		
61	27.2	11.45		
62	35.8	nd		
63	22.8	11.45		
64	16.98	8		
65	19.96	16.45		
66	19.51	8.45		
67	24.77	12.15		
68	29.2	28.5		
69	32.3	nd		
70	26.6	20.3		
71	33.1	28		
72	20	7.3		
73	18.6	8.3		
74	17.5	7.5		
75	30.7	25.3		
76	20.8959	13		
77	18.9623	7.15		
78	26.3013	11.45		
79	22.5565	8.3		
80	29.298	nd		
81	20.8656	8.3		
82	21.45	14.15		
83	20.02	7.45		

84	26.58	nd	
85	28.48	nd	
86	28.21	26.15	
87	30.27	nd	
88	27.63	13.15	
89	20.03	9.15	
90	27.444	27.45	
91	18.65	7.15	
92	29.971	20.50	
93	28.036	nd	10
94	33.232	nd	nd
95	19.745	8.45	
96	28.239	nd	17.45
97	34.66	9.00	
98	25.78	nd	25.3
99	21.28	12.15	
100	16.53	7.45	
101	33.44	25.15	
102	25.13	19.00	
103	20.26	9.75	
104	26.82	nd	12.25
105	25.11	16.00	
106	22.39	12.50	
107	21.39	8.25	

Sample	E-gene RT-qPCR Ct	N1-STOP-LAMP Tp				Agreement with RT-qPCR
		Lab A	Lab B	Lab C	Lab D	
1	16.3	6.31	8	5.2	4.8	4/4
2	nd	nd	nd	19.9	nd	3/4
3	16.5	6.54	4.442	6.1	4.9	4/4
4	nd	nd	nd	nd	nd	4/4
5	20.4	8.51	8.74	8.2	7.4	4/4
6	nd	nd	nd	nd	nd	4/4
7	21.7	9.3	5.98	7.2	6.9	4/4
8	nd	nd	nd	nd	nd	4/4
9	22.1	12.3	12.1	11.6	7	4/4
10	nd	nd	nd	nd	nd	4/4
11	22.8	8.45	5.31	7.4	7.9	4/4
12	25.4	16.45	16.28	9.5	7.1	4/4
13	25.9	24.3	26.35	20.8	25.2	4/4
14	27.2	nd	24.54	nd	nd	1/4
15	27.6	29.15	25.3	23.3	28.1	1/4
16	28.5	29.15	nd	nd	nd	1/4
17	29	nd	nd	nd	nd	0/4
18	30.6	29.15	23.07	nd	24	3/4
19	nd	nd	nd	nd	nd	4/4
20	nd	nd	nd	nd	nd	4/4

577 **Supplementary Table S2:** Results of N1-STOP-LAMP EQA among four laboratories

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591 **Supplementary Table S3:** N1-STOP-LAMP screen of NATtrol™ Respiratory Panel 2 (RP2) Controls

Virus/Organism	Strain	RP2 Control1	RP2 Control2	N1-STOP-LAMP
Adenovirus Type 1	N/A	Positive	Negative	Negative
Adenovirus Type 3	N/A	Positive	Negative	Negative
Adenovirus Type 31	N/A	Positive	Negative	Negative
C. pneumoniae	CWL-029	Positive	Negative	Negative
Influenza A 2009 H1N1pdm	A/NY/02/2009**	Positive	Negative	Negative
Influenza A 2009 H3N2	A/Brisbane/10/07	Positive	Negative	Negative
Human Metapneumovirus Type 8	Peru6-2003	Positive	Negative	Negative
M. pneumoniae	M129	Positive	Negative	Negative
Parainfluenza Type 1	N/A	Positive	Negative	Negative
Parainfluenza Type 4	N/A	Positive	Negative	Negative
Rhinovirus Type 1A	N/A	Positive	Negative	Negative
B. parapertussis	A747	Negative	Positive	Negative
B. pertussis	A639	Negative	Positive	Negative
Coronavirus 229E	N/A	Negative	Positive	Negative
Coronavirus HKU-1	Recombinant	Negative	Positive	Negative
Coronavirus NL63	N/A	Negative	Positive	Negative
Coronavirus OC43	N/A	Negative	Positive	Negative
Influenza A H1N1	A/New Cal/20/99	Negative	Positive	Negative
Influenza B	B/Florida/02/06	Negative	Positive	Negative
Parainfluenza Type 2	N/A	Negative	Positive	Negative
Parainfluenza Type 3	N/A	Negative	Positive	Negative
RSV Type A	2006 isolate	Negative	Positive	Negative

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597 **Supplementary data file 1:** GISAID submitters table

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