

1 **Comparative performance of four nucleic acid amplification tests for**
2 **SARS-CoV-2 virus**

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18

19 **Abstract**

20 Coronavirus disease 2019 (COVID-19) can be screened and diagnosed through the
21 detection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by
22 real-time reverse transcription polymerase chain reaction. SARS-CoV-2 nucleic acid
23 amplification tests (NAATs) have been rapidly developed and quickly applied to
24 clinical testing during the pandemic. However, studies evaluating the performance of
25 these NAAT assays are limited. We evaluated the performance of four NAATs, which
26 were marked by the Conformité Européenne and widely used in China during the
27 pandemic. Results showed that the analytical sensitivity of the four assays was
28 significantly lower than that claimed by the NAAT manufacturers. The limit of
29 detection (LOD) of Daan, Sansure, and HybriBio NAATs was 3000 copies/mL,
30 whereas the LOD of Bioperfectus NAATs was 4000 copies/mL. The results of the
31 consistency test using 46 samples showed that Daan, Sansure, and HybriBio NAATs
32 could detect the samples with a specificity of 100% (30/30) and a sensitivity of 100%
33 (16 /16), whereas Bioperfectus NAAT detected the samples with a specificity of 100%
34 (30/30) and a sensitivity 81.25% (13/16). The sensitivity of Bioperfectus NAAT was
35 lower than that of the three other NAATs; this finding was consistent with the result
36 that Bioperfectus NAAT had a higher LOD than the three other kinds of NAATs. The
37 four above mentioned reagents presented high specificity; however, for the detection
38 of the samples with low virus concentration, Bioperfectus reagent had the risk of
39 missing detection. Therefore, the LOD should be considered in the selection of
40 SARS-CoV-2 NAATs.

41

42 Key words: Severe acute respiratory syndrome coronavirus 2; SARS-CoV-2; nucleic
43 acid amplification test; performance evaluation; Coronavirus disease 2019;
44 COVID-19.

45

46 **Introduction**

47 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread rapidly
48 since its recent identification in patients with severe pneumonia in Wuhan, China(1-3).
49 To date, SARS-CoV-2 has affected more than 372,000 patients worldwide and
50 resulted in more than 16,000 deaths (as of March 24, 2020)(4). The World Health
51 Organization (WHO) has declared the Chinese outbreak of coronavirus disease 2019
52 (COVID-19) to be a Public Health Emergency of International Concern and stated
53 that the spread of COVID-19 may be interrupted by early detection, isolation, prompt
54 treatment, and the implementation of a robust system(4, 5). On March 11, at a regular
55 press conference in Geneva, WHO Director-General Tan Desai said that the
56 COVID-19 epidemic can be called a pandemic in terms of its characteristics.
57 Unfortunately, no drug or vaccine has yet been approved to treat human coronaviruses,
58 and new interventions are likely to require months to years to develop(6). Therefore,
59 early diagnosis of SARS-CoV-2 infection is important to distinguish it from
60 asymptomatic, healthy, and other pathogenic infections and to ensure timely isolation
61 of the infected patients.

62
63 At present, various detection methods for SARS-CoV-2 infection have been reported,
64 such as real-time reverse transcription–polymerase chain reaction (RT-PCR) assay,
65 sequencing, CRISPR technique, nucleic acid mass spectrometry, and serum
66 immunology(7). However, real-time RT-PCR remains the primary means for
67 diagnosing COVID-19 among the various diagnostic platforms available. The
68 development of RT-PCR methods to diagnose COVID-19 mainly targets various
69 combinations of the open reading frame (ORF), envelope (E), nucleocapsid (N), spike
70 (S), and RNA-dependent RNA polymerase (RdRp) genes(8-10).

71
72 Although real-time RT-PCR assay is a highly sensitive technique, false negative
73 results have still been reported in some COVID-19 patients. These results may occur
74 due to insufficient organisms in the specimen resulting from improper collection,
75 transportation, storage, and handling, as well as laboratory test conditions and

76 personnel operation(11-13). Moreover, the quality of the examined reagents is related
77 closely to the PCR results for SARS-CoV-2. In response to this outbreak, a number of
78 nucleic acid amplification tests (NAATs) for SARS-CoV-2 were rapidly developed in
79 China and quickly applied to clinical testing. To date, no quality evaluation of
80 SARS-CoV-2 NAATs has been reported.

81

82 The purpose of this study was to comprehensively evaluate the performance of four
83 SARS-CoV-2 NAATs, which were Conformité Européenne (CE) marked and widely
84 used. The results of this study will be helpful for laboratorians to select the
85 appropriate assay for detecting SARS-CoV-2.

86

87 **Materials and methods**

88 **Tested samples.** The kit of performance verification reference materials, which were
89 cell cultures containing pseudoviruses, included 10 positive reference materials, two
90 negative reference materials, 18 analytical specificity reference materials, and three
91 interference reference materials (Guangzhou BDS Biological Technology Company
92 Limited).The positive quality control agents were made with cell cultures containing
93 SARS-CoV-2 pseudoviruses (Guangzhou BDS Biological Technology Company
94 Limited).The pseudoviruses in the positive quality control agents included segments
95 of the ORF1ab gene (the genome coordinates: 900–1500,12200–13500, 18770–18950,
96 and 20560–24453), N gene, and E gene, which were the important characteristic
97 genes of SARS-CoV-2. RNA transcripts, presented by the Chinese Academy of
98 Metrology, were used as standard materials. The RNA transcripts obtained the
99 complete N gene, the complete E gene, and ORF1ab gene segment (14911–15910,
100 GenBank No. NC_O45512) of SARS-CoV-2. The standard value of reference
101 material was obtained by absolute quantitative digital PCR. RNA was extracted from
102 cell cultures with the nucleic acid extraction reagent (Tianlong Technology Company
103 Limited), aliquoted into multiple tubes for testing with each NAAT method, and
104 stored at –80 °C.

105 Human samples (n = 46), which were RNA leftovers from daily laboratory activity,

106 were provided by the Guangdong Provincial Hospital of Chinese Medicine and
107 HybriBio Medical Laboratory. The selection of clinical samples used in the study was
108 exclusively based on their SARS-CoV-2 virus detection result. The study was
109 approved by the Ethics Committees from the Guangdong Provincial Hospital of
110 Chinese Medicine (ZE2020-027-01).

111

112 **Assay and equipment.** Four sets of systems were used to detect the SARS-CoV-2
113 virus: Daan SARS-CoV-2 NAAT on LightCycler 480 II PCR instrument (Daan
114 NAAT), Sansure SARS-CoV-2 NAAT on SLAN-96P PCR instrument (Sansure
115 NAAT), HybriBio SARS-CoV-2 virus NAAT on SLAN-96P PCR instrument
116 (HybriBio NAAT), and Bioperfectus SARS-CoV-2 NAAT on SLAN-96P PCR
117 instrument (Bioperfectus NAAT). Real-time PCR was applied to the four systems,
118 which targeted the combinations of the ORF1ab gene, N gene of SARS-CoV-2, and
119 endogenous gene of humans. About 5 μ L of RNA was added to the PCR mixture and
120 tested according to the manufacturers' instructions for each assay. Positive results
121 were defined as the simultaneous detection of the ORF1ab gene and N gene. The four
122 systems had different detection sites for the ORF1ab gene. The PCR primers designed
123 for the four systems were complementary to the sites on the SARS-CoV-2 genomic
124 sequence (GenBank No. NC_045512) in the region of 20700–21000 (Daan),
125 12500–13300 (Sansure), 13400–13550 (Bioperfectus), and 7100–7600 bp (HybriBio).
126 Cycle threshold (Ct) values (i.e., number of cycles required for the fluorescent signal
127 to cross the threshold in RT-PCR) quantified viral load, with low values indicating a
128 high viral load.

129

130 **Analytical Sensitivity.** Analytical sensitivity was examined in accordance with a
131 modified EP17-A protocol, MM03 and MM19-A guidelines of Clinical Laboratory
132 Standards Institute (CLSI). The analytical sensitivity of each NAAT assay was
133 compared using the same RNA samples obtained from the extraction of mixed clinical
134 samples or positive quality controls. The concentrations of RNA samples were
135 determined by fluorescence quantitative method using standard materials as standard.

136 Serial dilutions were made with a known concentration of the target substance in the
137 analytical range of the expected limit of detection (LOD) and tested in replicates of 20.
138 All panel members were prepared at the same time, aliquoted into individual tubes for
139 each concentration and each assay, stored frozen, and thawed on the day of testing.
140 When 19 or all 20 replicates were positive, the concentration was temporarily defined
141 as LOD, followed by 20 replicates per day for two consecutive days, yielding a total
142 of 60 results. If more than 95% of the results were positive, the concentration was
143 finally determined as the LOD of the reagent. The experiments were spread over 3
144 days so that the standard deviations reflect the performance of the assay over a range
145 of typical laboratory conditions but without a change in reagent lots.

146

147 **Precision.** Precision was evaluated in accordance with a modified EP12 protocol,
148 MM09 and MM19-A guideline of CLSI using a patient RNA pool with concentration
149 above 20% of the LOD and the negative sample. Sample pools were divided into five
150 aliquots per level and frozen at -70°C . One aliquot of each level was thawed daily
151 and analyzed four times per day during a period of five consecutive workdays ($n = 20$
152 per level). Precision was evaluated as the coefficient of variation (CV), which was
153 calculated from the data series mean and standard deviation.

154

155 **Accuracy.** Ten positive reference materials and two negative reference materials from
156 the performance verification reference material kit were used to evaluate accuracy.

157

158 **Analytical specificity.** Eighteen pseudovirus samples of analytical specificity
159 reference materials from the performance verification reference material kit were used
160 to determine the analytical specificity of four NAATs, including human coronavirus
161 HCoV-OC43, HCoV-HKU1 RNA, HCoV-229E RNA, HCoV-NL63 RNA, SARS
162 RNA, Middle East respiratory syndrome (MERS) RNA, influenza A H1N1 virus,
163 influenza B INFB virus, respiratory syncytial virus type A and type B, human
164 parainfluenza virus, adenovirus, enterovirus, mycoplasma pneumoniae, Epstein–Barr
165 (EB) virus, human cytomegalovirus, *Mycobacterium tuberculosis*, and two samples

166 with human genome DNA.

167

168 **Analytical interferences.** To investigate the analytical interferences, three
169 interference reference materials from the performance verification reference material
170 kit were used, including 6 g/dL hemoglobin, 30 g/dL albumin, and the mix of 100
171 µg/mL ribavirin and 100 µg/mL azithromycin. All tests were repeated three times.

172

173 **Method comparison.** Forty-six RNA samples from suspected patients with
174 COVID-19 were used in the method comparison. Given that no reference method was
175 used to determine the sensitivity and specificity of NAATs, the consensus result was
176 determined for each sample; samples were categorized as true positive (TP) if positive
177 by two or more assays (14). The SARS-CoV-2-positive samples were further
178 confirmed by sequencing.

179

180 **Statistical analysis.** The significance of the difference in sensitivity between the
181 NAATs was assessed by using Fisher's exact test in GraphPad Prism (5.0). The
182 Chi-square likelihood ratio test was used to analyze the discordant results. The 95%
183 confidence intervals (CIs) for specificity and sensitivity were calculated by using the
184 Wilson–Score method in GraphPad Prism. Significant differences were considered at
185 *P* values less than 0.05.

186

187 Result

188 **Analytical sensitivity.** We evaluated the analytical sensitivity of each assay to
189 determine differences in the ability of each assay to detect SARS-CoV-2 in mixed
190 clinical samples or in pseudovirus cultures. First, we contrived RNA specimens from
191 SARS-CoV-2 pseudovirus cultures at four separate final concentrations (500, 1000,
192 2000, and 3000 copies/mL). The ORF1ab gene test results of the serial dilutions were
193 all negative by the HybriBio and the Bioperfectus NAATs. Further investigation
194 revealed that the pseudoviral RNA did not contain the detecting locus of HybriBio and
195 Bioperfectus NAATs targeting the ORF1ab gene. Daan NAAT detected 18/20

196 replicates at 2000 copies/mL and 20/20 replicates at 3000 copies/mL. Sansure NAAT
197 detected 17/20 at 1000 copies/mL and 20/20 at 2000 copies/mL (Table 1). The final
198 results of 60 replicates showed that the LOD of Daan NAAT was 3000 copies/mL for
199 pseudovirus cultures, and the LOD of Sansure NAAT was 2000 copies/mL for
200 pseudovirus cultures.

201

202 To explore the LOD of the NAATs in clinical samples, we further contrived mixed
203 RNA specimens from clinical samples at three separate final concentrations (2000,
204 3000, and 4000 copies/mL). Daan NAAT detected 12/20 replicates at 2000 copies/mL
205 and 20/20 replicates at 3000 copies/mL (Table 1). Sansure NAAT detected 17/20 at
206 2000 copies/mL and 20/20 at 3000 copies/mL. HybriBio NAAT detected 10/20
207 replicates at 2000 copies/mL and 20/20 replicates at 3000 copies/mL. Bioperfectus
208 NAAT detected 10/20 at 3000 copies/mL and 20/20 at 4000 copies/mL. The final
209 results of 60 replicates showed that the LOD of clinical samples was 3000 copies/mL
210 by Daan, Sansure, and HybriBio NAATs, whereas that of clinical samples was 4000
211 copies/mL by Bioperfectus NAAT. The LODs declared in the instructions of Daan,
212 Sansure, HybriBio, and Bioperfectus NAATs were 500, 200, 1000, and 1000
213 copies/mL, respectively.

214

215 **Precision.** For the negative sample, the 20 repeated test results by all four NAATs had
216 no amplification curve of target genes but a positive amplification curve of the
217 internal reference gene. For the positive sample, 20 repeated test results by all four
218 NAATs had positive amplification curves of the target genes and internal reference
219 gene. The within-run CVs at the concentrations above the 20% level of the LOD on
220 all four systems were less than 4%, and the total CVs were less than 5% (Table 2).

221

222 **Accuracy.** The results of all four NAATs for accuracy evaluation were in line with
223 expectations by using 10 positive reference materials and two negative reference
224 materials.

225

226 **Analytical specificity.** For the four assays, no amplification curves were obtained for
227 up to 10,000 copies/mL of six coronavirus pseudovirus samples with HCoV-OC43,
228 HCoV-HKU1, HCoV-229E, HCoV-NL63, SARS, and MERS RNA. For the 10
229 common respiratory pathogens and two human genomic DNA samples, all results of
230 the four assays were also negative.

231

232 **Analytical interferences.** An assessment of interferences is shown in Table 3. The
233 bias of Ct value for the N gene and ORF1ab gene on all four systems was less than
234 8.5% at the following interfering substance concentrations tested: 6 g/dL hemoglobin,
235 30 g/dL albumin, 100 µg/mL ribavirin, and 100 µg/mL azithromycin.

236

237 **Comparison of the four NAATs.** We defined consistency as two or more tests that
238 produce same results and used these results to define true positives and negatives. By
239 this definition, 34.8% (16/30) tested positive and 65.2% (30/46) tested negative. The
240 results for all four NAATs were negative for all 30 negative samples, and the 16
241 positive samples were reported positive by Daan, Sansure, and HybriBio NAATs.
242 However, three of the 16 positive samples were reported negative by Bioperfectus
243 NAAT (Table 4). The Ct values of the N gene and ORF1ab gene in the three positive
244 samples by Daan, Sansure, and HybriBio NAATs are shown in Table 5, and the Ct
245 values of the ORF1ab gene were close to the positive threshold of the three NAATs
246 declared in their instructions.

247

248 The sensitivity and specificity for each NAAT were calculated based on consistent
249 analysis. For Daan, Sansure, and HybriBio NAATs, their specificity and sensitivity
250 were 100%. For Bioperfectus NAAT, the sensitivity was 81.25% and specificity was
251 100%. No significant difference was found in the sensitivity between Bioperfectus
252 NAAT and the three other NAATs ($P > 0.05$).

253

254 **Discussion**

255 The prevention of the COVID-19 epidemic is very grim. On the basis of China's

256 experience in preventing the further spread of the disease in the past 2 months,
257 identifying COVID-19 from patients with other diseases as soon as possible has been
258 proven to be particularly important to execute early isolation and early treatment (4, 5,
259 15). The use of RT-PCR technology to detect SARS-CoV-2 nucleic acid is an
260 effective means to screen SARS-CoV-2 virus-infected patients. The current
261 SARS-CoV-2 NAATs, which are widely used in China, were developed in response to
262 the emergency situation of screening for SARS-CoV-2 infection. The performance of
263 the assays is exactly what all testers want to know. In this study, four NAATs were
264 evaluated. The results showed that the LODs of the four assays were significantly
265 higher than the LODs declared in their instructions, suggesting that each laboratory
266 should re-evaluate the LOD of the reagents depending on the needs of their laboratory.
267 The RNA detection data from pseudovirus cultures showed that the LOD of Sansure
268 was slightly lower than that of Daan NAAT. The RNA detection data from clinical
269 samples showed that the LOD of Bioperfectus NAAT was slightly higher than that of
270 the three other reagents but still reached the detection sensitivity of 20 copies per PCR
271 reaction. There was no significant difference in the LOD of SARS-CoV-2 virus
272 between pseudovirus cultures and clinical samples for Daan and Sansure NAATs.
273 Thus, pseudovirus cultures could be used as an alternative for clinical samples in the
274 performance evaluation of LOD. In addition, the four assays showed high precision,
275 and the CV value was less than 5% when the concentration was 20% higher than the
276 LOD. There were no cross-reactions by the four assays with four common human
277 coronaviruses, SARS, and MERS viruses and no cross-reaction with other common
278 respiratory pathogens, such as respiratory syncytial virus, adenovirus, influenza virus,
279 and *Mycoplasma pneumoniae*.

280

281 In the present study, we further compared four NAATs for the detection of
282 SARS-CoV-2 in clinical samples from suspected COVID-19 patients. Results showed
283 that the clinical sensitivity and specificity of Daan, Sansure, and HybriBio NAATs
284 were 100%, while three of the positive samples were missed by Bioperfectus NAAT.
285 The Ct values of the ORF1ab gene of the three samples tested with Daan, Sansure,

286 and HybriBio reagents were all close to 40, indicating that the concentration of the
287 three samples may be lower than the LOD of Bioperfectus. Therefore, the LOD
288 should be included in the reference index when selecting reagents for SARS-CoV-2 in
289 the laboratory. Sequencing verification of the PCR-positive samples in this study
290 suggested that the four assays did not show false positives in the detection of limited
291 clinical samples. Although the small clinical sample size limited the optimal
292 evaluation of the performance of these assays, the results of this study can still help
293 laboratories to select an appropriate SARS-CoV-2 NAAT supplier and interpret their
294 detection results.

295

296 Obtaining high-quality viral RNA from original clinical samples is crucial to ensure
297 the accuracy of the detection results by NAATs. Therefore, the poor quality of
298 extraction reagents and the unoptimized extraction process of viral RNA would affect
299 the detection results. In this study, the performance of NAATs was evaluated by using
300 the same viral RNA samples. Therefore, the difference in the experimental results
301 only reflected the difference in the performance of nucleic acid amplification for
302 NAATs but not the diversity in the performance of the whole SARS-CoV-2 detection
303 system, which usually includes the nucleic acid extraction system.

304

305 In addition, RNA viruses may show substantial genetic variability. This could result in
306 mismatch between the primer and probes with the target sequence, which can
307 diminish the assay performance or result in false negative results. Therefore, the
308 manufacturer of NAATs should pay attention to the sequence update information of
309 the virus database at any time to ensure no mutation exists in the genome sequences
310 corresponding to the primers, especially for the sequences corresponding to the 3' end
311 of the primers.

312

313 In summary, the four SARS-CoV-2 NAATs commonly used with commercial assays
314 showed good reliability, but the actual LOD was significantly higher than the declared
315 LOD. The analytic sensitivity of Daan, Sansure, and HybriBio NAATs was slightly

316 higher than that of Bioperfectus NAAT.

317

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321

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371

372

373 **Table 1.** Comparison of Analytical sensitivity.

Copies/mL	Pseudoviruses				Clinical samples			
	Daan	Sansure	HybriBio	Bioperfectus	Daan	Sansure	HybriBio	Bioperfectus
4000	/	/	/	/	20/20	20/20	20/20	20/20
3000	20/20	20/20	0/20	0/20	20/20	20/20	20/20	12/20
2000	18/20	20/20	0/20	0/20	12/20	17/20	17/20	5/20
1000	12/20	17/20	0/20	0/20	/	/	/	/
500	5/20	17/20	0/20	0/20	/	/	/	/

374

375 **Table 2.** Comparison of precision.

	N gene				ORF1ab gene					
	Standard	CV (%)	Within-run	CV (%)	Total	Standard	CV (%)	Within-run	CV (%)	Total
Daan	39.87	0.54		0.64		37.22	1.60		4.18	
Sansure	36.20	2.40		2.41		38.41	3.12		3.55	
HybriBio	35.27	1.71		1.95		37.03	2.79		2.96	
Bioperfectus	33.90	3.16		3.24		33.33	2.25		3.59	

376

377 **Table 3.** Sample interferences for common interferents.

Interferent	Relative bias from native sample, Ct%							
	Daan		Sansure		HybriBio		Bioperfectus	
	N	ORF1ab	N	ORF1ab	N	ORF1ab	N	ORF1ab
Hemoglobin	7.29	5.96	6.06	2.84	7.45	7.00	7.20	4.15
Albumin	6.72	5.92	6.83	3.39	8.22	7.96	7.20	3.72
Ribavirin & Azithromycin	6.72	5.92	6.76	4.54	7.86	7.60	7.20	4.23

378

379 **Table 4.** Comparison of NAATs to consensus results for detection of SARS-CoV-2.

Assays	TP	TN	FP	FN	Sensitivity	Specificity
					(95% CI)	(95% CI)
Daan	16	30	0	0	100%(80.64 - 100)	100%(80.64 - 100)
Sansure	16	30	0	0	100%(80.64 - 100)	100%(80.64 - 100)
HybriBio	16	30	0	0	100%(80.64 - 100)	100%(80.64 - 100)
Bioperfectus	13	30	0	3	81.25%(56.99 - 93.41)	100%(88.65 - 100)

380

381 **Table 5.** Ct values of the three clinical samples which was SARS-CoA-2 negative detected by
382 Bioperfectus NAATs.

Samples	Daan		Sansure		HybriBio	
	N	ORF 1ab	N	ORF 1ab	N	ORF 1ab
S1	34.57	38.09	37.22	38.48	36.27	38.51
S2	35.45	40	37.09	40	37.96	39.69
S3	35.83	40	36.16	39.97	36.24	37.42

383