

1 Chimeric synthetic reference standards enable cross- 2 validation of positive and negative controls in SARS- 3 CoV-2 molecular tests.

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17 ABSTRACT

18 DNA synthesis *in vitro* has enabled the rapid production of reference standards. These are used as
19 controls, and allow measurement and improvement of the accuracy and quality of diagnostic tests.
20 Current reference standards typically represent target genetic material, and act only as positive controls
21 to assess test sensitivity. However, negative controls are also required to evaluate test specificity. Using a
22 pair of chimeric A/B RNA standards, this allowed incorporation of positive and negative controls into
23 diagnostic testing for the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). The chimeric
24 standards constituted target regions for RT-PCR primer/probe sets that are joined in tandem across two
25 separate synthetic molecules. Accordingly, a target region that is present in standard A provides a positive
26 control, whilst being absent in standard B, thereby providing a negative control. This design enables cross-
27 validation of positive and negative controls between the paired standards in the same reaction, with
28 identical conditions. This enables control and test failures to be distinguished, increasing confidence in
29 the accuracy of results. The chimeric A/B standards were assessed using the US Centers for Disease
30 Control real-time RT-PCR protocol, and showed results congruent with other commercial controls in
31 detecting SARS CoV-2 in patient samples. This chimeric reference standard design approach offers
32 extensive flexibility, allowing representation of diverse genetic features and distantly related sequences,
33 even from different organisms.

38 **INTRODUCTION.**

39 Reference standards are required to validate the performance of any diagnostic test [1]. The recent advent
40 of DNA synthesis enables the rapid development of reference standards as synthetic constructs
41 representing target genetic material. These can be used as positive controls to assess sensitivity of the
42 molecular test undergoing evaluation. However, separate negative controls, without the target
43 sequences, are also required to ensure the specificity of the diagnostic test. Reference standards must be
44 validated and proven fit-for-purpose before used in diagnostic tests. In the case of RNA standards, the
45 synthetic controls undergo degradation over time, and can be contaminated, confounding the
46 interpretation of test results. However, this failure of either positive or negative controls is difficult to
47 distinguish from the failure of the diagnostic test itself. For example, if the test returns a negative result
48 from the positive control, it could be because (i) the test failed, (ii) the reference control failed or (iii) a
49 technical issue with the testing platform. This leads to delays in diagnosis, missed diagnoses and
50 invalidation of correct test results.

51 The use of *in vitro* synthesis of RNA and DNA standards allows flexibility in control design and tailoring of
52 controls to the diagnostic test and targets. Here, we propose a new design strategy for reference
53 standards that uses matched chimeric synthetic standards in accordance with the principle of A/B testing.
54 In this design, all the target sequences of a molecular test are retrieved and split between groups A and
55 B, which are then joined in tandem to form single chimeric sequences A and B. This means that for each
56 target used in the molecular test, standard A would act as positive control, while standard B would act as
57 negative control, or vice-versa. Furthermore, the equally partitioning of target sites between standards A
58 and B enables cross-validation of positive and negative controls, increasing the confidence in test results.
59 Among the benefits of this design, a chimera allows concurrent testing of disparate target regions of a
60 single pathogen or even different organisms and splitting targets between standards A and B enables
61 control cross-validation, facilitating the distinction of control failure from test failure or success.

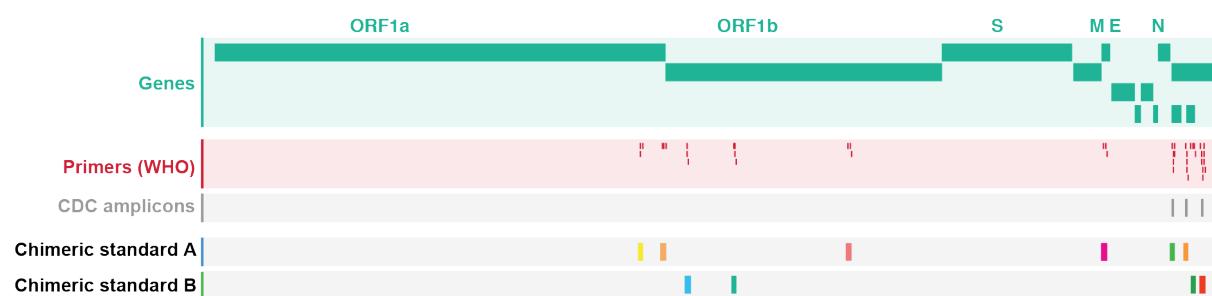
62 The recent emergence of the SARS-CoV-2 pandemic has required widespread diagnostic testing for active
63 virus infections, including genome sequencing, but predominantly using real-time reverse-transcriptase
64 polymerase chain reaction (real-time RT-PCR)-based assays [2-4]. The World Health Organisation (WHO)
65 published seven diagnostic testing protocols for detection of SARS-CoV-2 that have been rapidly adopted
66 worldwide, with over 20 million molecular tests performed globally by mid-2020 [5, 6]. These tests
67 typically employ multiple primer pairs homologous with SARS-CoV-2 genes E, N, Orf 1a/1b and RdRp [7-
68 9]. Diagnosis is considered positive if all targets are amplified or presumptive positive if some but not all
69 targets are detected. In addition, some tests contain primer pairs also targeting human genes as internal
70 positive controls to ensure sample quality.

71 We used a pair of chimeric A/B standards for the WHO-endorsed real-time RT-PCR tests to demonstrate
72 the utility of the chimeric A/B approach to designing reference test standards. Each standard included
73 regions of the coronavirus genome (SARS-CoV-2) that are targeted by published primer pairs. As a result,
74 it is compatible with endorsed diagnostic tests licensed globally. We compared the performance of the
75 synthetic controls to other reference materials and patient samples, and demonstrated how the two
76 synthetic RNA standards can be used to validate the standard real-time RT-PCR test (CDC), and also
77 considered the utility of these standards in other assays [10].

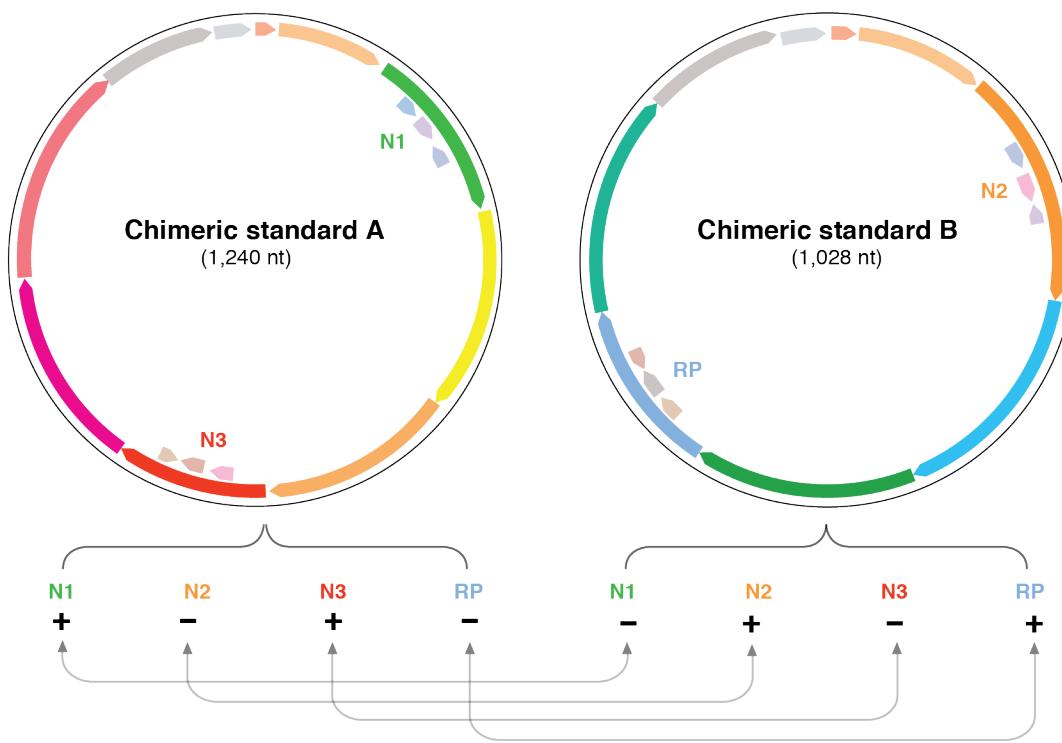
78

79 **RESULTS.**

(a) SARS-CoV-2 Reference Genome (Wuhan-Hu-1, NC_045512.2)



(b) Chimeric control design.



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81

82 **Figure 1. Design of chimeric controls for SARS-CoV-2. (a)** Genome browser view of the SARS-CoV-2 genome (green) showing WHO-published real-time RT-PCR primer binding sites (red). The expected amplicons for the CDC test are shown in darker grey. The other targeted regions were exclusively partitioned between chimeric A/B standards. **(b)** The different targeted regions for standards A and B were shuffled and joined together to form chimeric sequences. The paired design of chimeric A/B standards, where a target in A is absent in B (and vice versa), enables the synthetic RNA transcripts to simultaneously act as positive and negative controls for the real-time RT-PCR primer/probe sets. This enables internal cross validation of positive and negative controls between standards A and B. The vector backbone was omitted from the representation of the chimeric A/B standards.

89

91 **Design of RNA standards.**

92 DNA synthesis enables rapid and flexible assembly of reference standards, including sequences not
93 present in natural organisms. This allows sequences from different genome regions to be aggregated to
94 address specific requirements in a diagnostic assay. To demonstrate this approach, we designed synthetic
95 reference sequences that encompass the primer binding sites of all WHO-published real-time RT-PCR
96 tests.

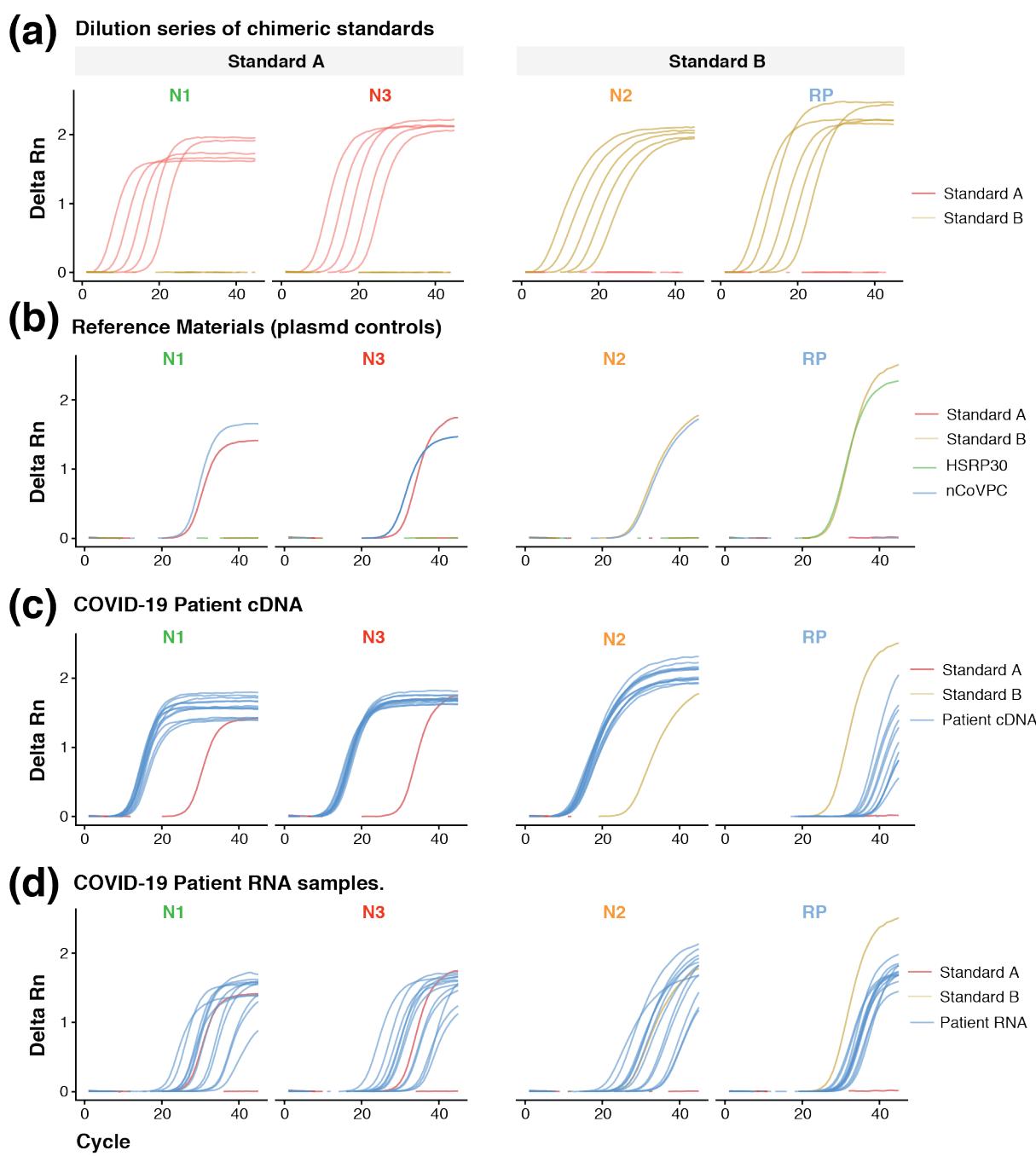
97 We first retrieved the SARS-CoV-2 genome sequence (isolate Wuhan-Hu-1, NC_045512.2), as well as the
98 primer sequences published by the World Health Organisation (WHO) for China, Hong Kong, Thailand,
99 United States (CDC), Germany and France (**Fig. 1a**). Each available real-time RT-PCR test typically
100 comprises 2-3 primer pairs that target different regions of the SARS-CoV-2 genome (**see Supplementary**
101 **Table 1**). We then aligned the primer pairs to the SARS-CoV-2 genome and identified the coordinates of
102 the amplicons, which were then retrieved along with an additional 30 nucleotides (nt) on either flanking
103 side (**Fig. 1a**).

104 We next organised these sequences across two different controls (termed chimeric A/B standards). We
105 partitioned the different targeted regions used by each country into two independent groups and then
106 assembled the regions in tandem (**Fig. 1b**). A fragment of the human RNase P gene (RP), which is used as
107 a positive human control, was also added to the chimeric standard B.

108 An additional unique control sequence (UCS) was also included at the 5' region of each standard to enable
109 the unique detection of the standards if required. Each standard sequence was then preceded by a T7
110 promoter to enable *in vitro* transcription, and followed by a poly-A tract (30nt length) and a restriction
111 site (EcoR1) to enable vector linearization.

112 The two distinct chimeric A/B standard sequences were then synthesised and cloned into pMK vector
113 backbones (**see Methods**). We then linearized the plasmids and performed *in vitro* transcription to
114 produce the synthetic RNA standards (**Fig. S1**). The resulting RNA transcript products were then purified
115 and validated (**see Methods** and **Supplementary Methods** for detailed protocol).

116 **Validation of chimeric RNA standards to alternative reference controls.**



117

118 **Figure 2. Real-time RT-PCR validation of chimeric A/B standards.** Amplification curves for the target
119 genes (N1, N2, N3 and RP) in the CDC real-time RT-PCR test for SARS-CoV-2. (a) The results for the chimeric
120 standards alone show that N1 and N3 were detected in standard A, but absent in B, while N2 and RP were
121 detected in standard B, but absent in A. (b) The chimeric standards achieve similar results to IDT controls,
122 which provide separate positive (nCoVPC) and negative controls (HSRP30). (c) Chimeric standards
123 compared to COVID-19 patient samples, where SARS-CoV-2 genome was previously amplified. (d)
124 Chimeric standards compared to RNA from confirmed COVID-19 RNA patient samples.

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127 We next validated the chimeric A/B standards by comparison to alternative reference controls. We first
128 performed real-time RT-PCR test using the established CDC primers and protocol [10]. Specifically, this
129 employs CDC primers (IDT) *N1*, *N2* and *N3* targeting the *N* gene from SARS-CoV-2 and the human *RNase P*
130 gene. Chimeric standard A includes regions of the *N1* and *N3* targets, while chimeric standard B includes
131 regions of the *N2* and *RP* targets. This allows for cross-validation between the chimeric A/B standards,
132 since the standards alternatively act as positive and negative controls to each primer/probe set in the
133 real-time RT-PCR test.

134 The real-time RT-PCR was initially performed on the chimeric controls alone. We prepared 10-fold
135 dilutions for each control, starting at 3.96×10^8 copies/ μ l for A and 4.22×10^8 copies/ μ l for B (see
136 **Methods**). As anticipated, in the reactions containing standard A, *N1* and *N3* primers returned positive
137 results, while *N2* and *RP* were undetected (**Fig. 2a**). In contrast, in reactions containing standard B, *N2* and
138 *RP* primers returned positive results, while *N1* and *N3* were undetected (**Fig. 2a**). In the real-time RT-PCR
139 reactions with positive results, there is an average increase in Ct values of 3.47 (sd= 0.34) for a 10-fold
140 dilution. These results show that the chimeric controls enable positive and negative cross-validation of
141 the published CDC real-time RT-PCR test for SARS-CoV-2.

142 We next performed the real-time RT-PCR test including commercial controls (Integrated DNA
143 Technologies Ltd.) for comparison with the chimeric A/B standards. The positive and negative commercial
144 controls are provided as separate plasmids. The positive control (2019-nCoV_N_Positive Control) contains
145 the complete nucleocapsid gene for SARS-CoV-2, while the negative control (Hs_RPP30) contains a
146 fragment of the human RNase P gene.

147 Therefore, IDT positive control, 2019-nCoV_N_Positive Control, should detect *N1*, *N2* and *N3*, but not *RP*,
148 while, IDT negative control, *Hs_RPP30*, should only detect *RP*. We diluted each plasmid to 4,000 copies/ μ l
149 along with the chimeric A/B standards and performed the real-time RT-PCR test with the CDC primers and
150 probes. The IDT controls worked as expected and the chimeric A/B standards achieved similar results (**Fig.**
151 **2b**). For each of the target genes, The Ct values were comparable between the IDT controls (N1=26;
152 N2=28; N3=28; RP=27) and the chimeric A/B standards (N1=27; N2= 27; N3= 30; RP=27).

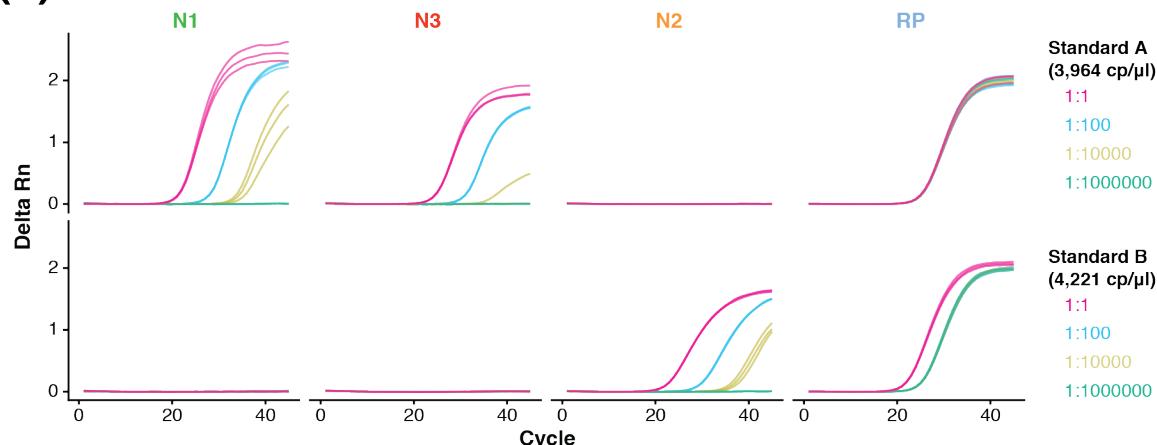
153 **Comparison of chimeric RNA standards COVID-19 patient samples.**

154 To validate the commutability of the chimeric A/B standards, we compared their performance in
155 amplifying genomes from 12 COVID patient samples that had been independently diagnosed and
156 sequenced (see **Methods**). These samples were available as raw total RNA containing the viral genome
157 and cDNA, where the viral genome was previously amplified. Therefore, the real-time RT-PCR test with
158 CDC primers should yield positive results for all target genes (*N1*, *N2*, *N3* and *RP*) in patient RNA samples
159 and primarily positive results for *N1*, *N2* and *N3* in patient cDNA samples.

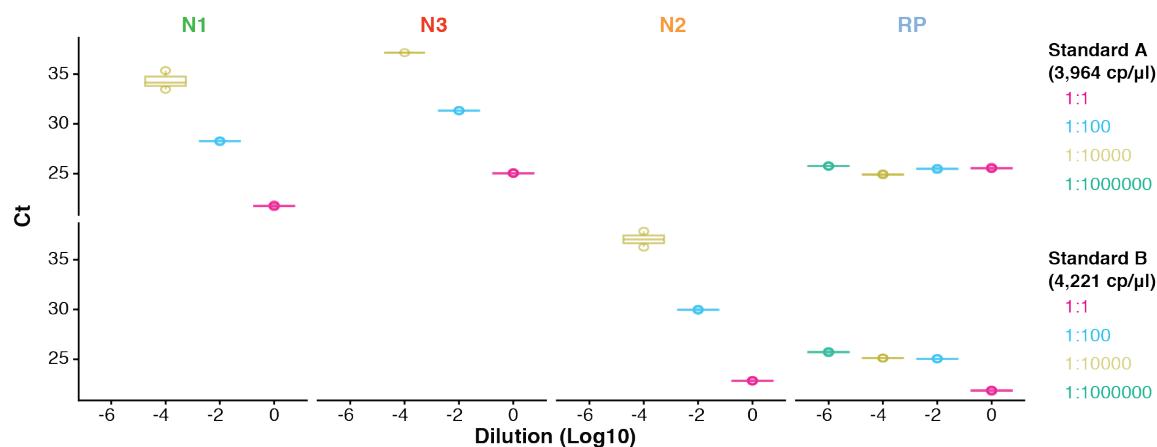
160 We diluted patient cDNA samples so that on average reactions had 2.79 ng (sd= 0.35) of input DNA. We
161 did not measure the concentration of patient RNA samples due to insufficient starting materials. The real-
162 time RT-PCR amplification results showed that both chimeric A/B standards and patient samples were
163 diagnosed as expected. Target genes *N1*, *N2* and *N3* were amplified in both patient cDNA (avg Ct; N1=12
164 \pm 0.72, N2=12 \pm 0.70, N3=13 \pm 0.69) and patient RNA (avg Ct; N1=29 \pm 4.85, N2=29 \pm 4.92, N3=29 \pm 4.89)
165 samples, with the former achieving significantly lower and less variable Ct values (**Figs. 2c,d**). However,
166 for the *RP* target gene, which is a positive control for human samples, the Ct value for patient cDNA was
167 significantly higher, since those samples are depleted of human material (37 \pm 1.83 and 31 \pm 1.54,
168 respectively).

169

(a) Amplification curves for serial dilution of chimeric A/B standards.



(b) Observed Ct values for serial dilution of chimeric A/B standards.



170

171 **Figure 3. Limit of detection of chimeric A/B standards.** (a) Amplification curves of targets N1, N2, N3 and RP in 100-fold serial dilutions of standards A and B against the human universal RNA, with three technical replicates. (b) Observed Ct values for targets N1, N2, N3 and RP at different dilutions of standards A and B (10^0 , 10^{-2} , 10^{-4} and 10^{-6}), across three technical replicates.

175

176 To determine the limit of detection (LoD) for the chimeric A/B standards, we performed 100-fold serial
177 dilutions with three technical replicates. As a baseline, we spiked the standards A (3,964 copies/μl) and B
178 (4,221 copies/μl) into separate background samples consisting of the human universal RNA (100 ng). We
179 then performed real-time RT-PCR using the CDC protocol (see **Methods**). As a result, we detected both
180 standards A and B until 10^{-4} dilution, which corresponds to approximate LoD of 0.39 and 0.42 copies/μl,
181 respectively (**Fig. 3a**). As a positive control, the RP primer targeting the human *RNase P* gene were
182 successfully detected in all tested dilutions, for both standards A and B (**Fig. 3b**). Interestingly, N1 primers
183 appear to be more efficient than N3 primers in estimating the LoD for standard A, since the Ct values for
184 N1 ($10^0=21.75 \pm 0.09$, $10^{-2}=28.26 \pm 0.04$ and $10^{-4}=34.33 \pm 0.96$) are significantly lower than N3 ($10^0=25.04$
185 ± 0.06 , $10^{-2}=31.33 \pm 0.04$ and $10^{-4}=37.16$), in every dilution, across replicates (**Fig. 3b**).

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187

188 **DISCUSSION.**

189 The advent of routine DNA synthesis has enabled rapid provision of synthetic reference standards that
190 can be used to validate the accuracy of diagnostic tests. The synthesis of DNA provides a flexible platform
191 to manufacture different reference standards, including non-natural designs. In this case, a single
192 standard can be designed to contain distant genomic regions or even sequences from different organisms.
193 This allows multiple sequences of interest to be included and organised within a single chimeric standard
194 according to the specific requirements of a diagnostic assay. In this study, we use this approach to
195 generate chimeric RNA transcripts that encode different SARS-CoV-2 genomic regions targeted by WHO
196 sanctioned molecular diagnostic tests.

197 Furthermore, we show how chimeric standards enables matched A/B testing of each primer/probe set
198 used in the real-time RT-PCR. Because the target regions are exclusively distributed between the A/B
199 standards, each inevitably functions as an independent positive or negative control for a given
200 primer/probe set. Additionally, the balanced distribution of target regions for each WHO sanctioned test
201 between A/B standards, ensures cross-validation of positive and negative controls. This improves the
202 confidence in the results of the controls and prevents a test failure being confused with a control failure.

203 Within this study we successfully validated the chimeric A/B standards using the CDC real-time RT-PCR
204 test for SARS-CoV-2 detection [10]. However, the chimeric A/B standards also contain the target sites in
205 accordance to official guidelines for molecular testing in China, Thailand, Hong Kong, Germany and France,
206 besides those of the United States. This means that the chimeric A/B standards can not only be used as
207 test controls in each of those countries individually, but also provide a common reference to compare test
208 results and the efficiency of their different primer/probe sets.

209 The Covid-19 pandemic has led to rapid development of novel diagnostic technologies. Design flexibility
210 in DNA synthesis enables the development of reference standards that simultaneously and specifically
211 address the validation requirements of a diverse range of diagnostic tests. We demonstrate the concept
212 with the chimeric A/B standards, but we anticipate this design approach can be used in applications
213 beyond SARS-CoV-2 diagnostic tests. For instance, a single synthetic control could represent multiple
214 genetic features, such as mutations, different haplotype blocks or viral sequences. This can also include
215 microbial sequences, cancer mutations and expressed gene signatures. In future testing of populations
216 with decreased incidence and prevalence of SARS CoV-2 infection and COVID-19 disease, internal controls
217 will be critical in allowing accurate and rapid assessment of infection. This is because the predictive value
218 of molecular and other testing is lower in low prevalence and low incidence populations. In summary, the
219 chimeric A/B approach provides a new model for the development of cost-effective reference standards
220 that allow for controlling of multiple experimental variables based on simple, but comprehensive designs.

221

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227 The contents of the published materials are solely the responsibility of the administering institution, a
228 participating institution or individual authors, and they do not reflect the views of the NHMRC or
229 CINSW.

230 **Author Contributions**

231 B.S.M, A.L.M.R. & T.R.M. conceived the project and devised the experiments. B.S.M conducted laboratory
232 experiments. I.W.D. and W.R. provided COVID-19 patient samples. A.L.M.R & T.R.M. performed data
233 analysis. A.L.M.R & T.R.M. prepared the manuscript, with support from all co-authors.

234 **Competing Interests**

235 The authors declare no competing interests.

236

237 **Ethics Declarations**

238 All procedures performed with human participants were in accordance with the ethical standards of
239 the Human Research Ethics Committee at South Eastern Sydney Local Health District (SESLHD), under
240 approval 2020/ETH00287. Where relevant, informed consent was obtained from individual research
241 participants.

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243

244 **MATERIALS AND METHODS**

245

246 **Covid-19 Patient samples**

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248 Patient samples used for validation tests were collected at the SAViD laboratories at Randwick as part of
249 a quality assurance study. The samples constitute viral RNA extracts (Roche MagNA Pure extraction kit)
250 on nasopharyngeal swabs from patients testing positive for SARS-CoV-2 infection. cDNA was generated
251 from the same isolates using Thermo Fisher Superscript IV VILO Master Mix, according to the
252 recommended protocol. cDNA was amplified with each of 14 x ~2.5 kb amplicons tiling the SARS-CoV-2
253 genome, according to a custom protocol [11]. Amplicons were then cleaned with AMPure beads and
254 pooled at equal abundance.

255

256 **Commercial controls**

257

258 We acquired control plasmids from IDT Technologies to be used in the CDC real-time RT-PCR diagnostic
259 assay of SARS-CoV-2. The positive control (2019-nCoV_N_Positive Control) contained the complete
260 nucleocapsid gene, while the negative control contains a portion of the human RPP30 gene. The stocks
261 for each of the plasmids were delivered at 200,000 copies/µl in IDTE pH 8.0. For the real-time RT-PCR, we
262 diluted the plasmids to 4,000 copies/µl each.

263

264

265 **Synthesis and preparation of chimeric standards**

266
267 The A/B standards were synthesized by a commercial vendor (ThermoFisher – GeneArt) and cloned into
268 pMK vectors. The plasmids containing the standards were each resuspended in 50 μ l nuclease free water
269 and transformed in *E. coli* as per manufacturer's protocol (α -Select Competent Cells, Bioline, Australia).
270 The transformed cells were grown overnight (37°) in LB agar plate containing Kanamycin (100 μ g/ml),
271 after which colonies were selected and further cultured overnight (37°; 200 rpm) in 3 ml LB broth also
272 containing Kanamycin (100 μ g/ml). The plasmids with the A/B standards were then extracted and purified
273 using the ZymoPURE Plasmid Miniprep Kit (Zymo Research), according to the manufacturer's protocol.
274 Purified plasmids were linearized by overnight digestion (37°) with EcoRI-HF (NEB) and the products were
275 then visualized on 1% agarose gel. The linear A/B standards were finally treated with Proteinase K and
276 further purified with the Zymo ChIP DCC-25 purification kit (Zymo Research). The final A/B standards were
277 quantified using Qubit dsDNA HS Assay on Qubit 2.0 Fluorometer (Life Technologies) and verified on the
278 Agilent TapeStation with the High Sensitivity DNA Screen Tape Analysis (Agilent Technologies).
279

280 ***In vitro* transcription**

281
282 The ChIP purified A/B standards were submitted to an *in vitro* transcription reaction, incubated overnight
283 at 37°, using the MEGAscript T7 Transcription kit (ThermoFisher) according to the manufacturer's
284 protocol. The resulting product was then treated with Turbo DNase and the remaining RNA was purified
285 with the Zymo RCC-25 column purification-25 kit (Zymo Research). The A/B RNA standards were
286 quantified using Qubit RNA HS Assay on Qubit 2.0 Fluorometer (Life Technologies) and then verified on
287 the Agilent TapeStation (Agilent Technologies) with the RNA ScreenTape Analysis (Agilent Technologies).
288

289 **Quantitative real time PCR**

290
291 Twenty μ l reactions were prepared containing 5 μ l of input RNA (patient samples, A/B standards or IDT
292 controls), 5 μ l of TaqPath™ 1-Step RT-qPCR Master Mix, 1.5 μ l of the combined CDC primers/probe set
293 and 8.5 μ l of Nuclease-free water. Thermo cycling was performed at 25° for 2 min to allow UNG
294 incubation, followed by 15 min at 50° for reverse transcription, then 2 min at 95° for enzyme activation
295 and finally 45 amplification cycles at 95° for 3 seconds and 55° for 30 seconds. The experiment was
296 performed on QuantStudio 7 Flex real-time PCR systems (Thermo Fisher).
297

298 **Dilution series**

299
300 We performed two different serial dilution experiments with the chimeric A/B standards. The first was a
301 10-fold serial dilution of standards A and B alone, to test their performance in the real-time RT-PCR assay.
302 The baseline concentration was 3.96×10^8 copies/ μ l for standard A and 4.22×10^8 copies/ μ l for standard
303 B. We diluted each standard until 10^{-5} . The second serial dilution was to estimate the limit of detection
304 (LoD) for the chimeric A/B standards. For this experiment, standards A and B were individually spiked into
305 universal human RNA samples. The baseline concentration was 3,964 copies/ μ l for standard A and 4,221
306 copies/ μ l for standard B and they were each added into 100 ng of universal human RNA. We made 100-
307 fold dilutions for the A/B standards until 10^{-6} , and the experiment was performed in three technical
308 replicates.
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