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1 **Sub-5-minute Detection of SARS-CoV-2 RNA using a Reverse Transcriptase-** 2 **Free Exponential Amplification Reaction, RTF-EXPAR**

3
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14
15 **We report a rapid isothermal method for detecting SARS-CoV-2, the virus responsible for**
16 **COVID-19. The procedure uses a novel reverse transcriptase-free (RTF) approach for**
17 **converting RNA into DNA, which triggers a rapid amplification using the Exponential**
18 **Amplification Reaction (EXPAR). Deploying the RNA-to-DNA conversion and amplification**
19 **stages of the RTF-EXPAR assay in a single step results in the detection of a sample of patient**
20 **SARS-CoV-2 RNA in under 5 minutes.**

21
22 In order to reduce the rate of spread of COVID-19, an accurate and efficient virus testing strategy is
23 imperative. A key part of this strategy is continuous assay development, with the aim of reducing
24 detection times and increasing sample throughput. The research community and diagnostics industry
25 has responded rapidly to this unprecedented crisis in developing a range of detection platforms.¹⁻⁴
26 The most sensitive assays detect viral RNA, with the current gold standard being reverse transcriptase
27 polymerase chain reaction (RT-PCR), a two-step assay that takes more than 60 minutes per sample.
28 First, reverse transcriptase converts viral RNA to complementary DNA (cDNA), a process that can
29 take up to 30 minutes.⁵ Then a quantitative PCR (qPCR) amplifies the cDNA, which is detected using
30 a fluorescent dye, a process that takes up to an hour.⁶ To reduce assay times, a plethora of new
31 approaches to SARS-CoV-2 detection have appeared in the literature over the past year. As far as
32 Nucleic Acid Amplification Tests (NAATs) are concerned, which are more sensitive than current 30-
33 minute lateral flow antigen (immunoassay) tests,⁷ focus has turned towards isothermal DNA
34 amplification approaches, which increase amplification speeds and hence reduce assay times. The
35 most common isothermal amplification system is Loop mediated isothermal AMplification (LAMP).⁸
36 LAMP assays have been developed for SARS-CoV-2 but take, on average, 20 minutes for a result,
37 with further decreases in LAMP assay time proving challenging.^{4,9,10} Herein, we demonstrate an
38 alternative isothermal approach based on the Exponential Amplification Reaction (EXPAR),¹¹ a
39 simpler and faster amplification method than LAMP. By combining EXPAR with a novel reverse
40 transcriptase-free (RTF) step, this new assay, RTF-EXPAR, can accurately identify viral RNA
41 derived from COVID-19 patient samples in less than 5 minutes.

42
43 The key to the speed of EXPAR is twofold; firstly, the amplification occurs at a single temperature,
44 thus avoiding lengthy heating and cooling steps, and secondly the amplicon is relatively small
45 (typically 15-20 bases long), compared to both PCR and LAMP. These two factors result in EXPAR,
46 once triggered, producing up to 10⁸ strands of DNA product in a matter of minutes.^{11,12} A single-
47 stranded DNA fragment (the trigger) starts the EXPAR reaction by binding a DNA template. Large
48 quantities of short double stranded DNA sequences are then generated in an isothermal cycle
49 involving a DNA polymerase to extend the sequence and a nicking endonuclease to cut it, while
50 leaving the template intact (Scheme 1a). As with the RT-PCR COVID-19 assay, duplex formation is
51 monitored spectroscopically using a fluorescent intercalating dye, e.g. SYBR Green.

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53 A crucial element to developing a successful EXPAR assay is the identification of optimal nucleotide
54 sequences in the target genome. Qian *et al* previously found that the type of trigger sequence used in
55 EXPAR plays a vital role in determining its efficiency.^{13,14} Using their approach, we designed a 17-
56 mer DNA trigger for EXPAR (**Trigger X**, Scheme 1a and Table 1) containing a sequence
57 complementary to one within the conserved gene *Orflab* in the SARS-CoV-2 genome
58 (<https://www.ncbi.nlm.nih.gov/nuccore/MN908947.3?report=fasta>). We first analysed the speed and
59 sensitivity of EXPAR using **Trigger X** in the presence of **Template X'-X'** (Supplementary Figure
60 1). Rapid rises in SYBR Green fluorescence were observed, with amplification times revealing an
61 expected dependence on trigger concentration (e.g. time to 10-sigma: 3.17 ± 0.14 minutes at 10 nM,
62 8.67 ± 1.08 minutes at 10 pM). These results demonstrate that EXPAR is a faster amplification
63 method than LAMP. Next, we analysed the specificity of the reaction by investigating three other
64 triggers (**Triggers A, B and C**), each at a concentration of 10 nM, that were non-complementary to
65 **Template X'-X'** (Supplementary Figure 2). Each of these three triggers produced no signal within
66 10 minutes under the same conditions, confirming the specificity of the EXPAR reaction, with only
67 the trigger sequence fully complementary to the template (**Trigger X**) resulting in rapid amplification.
68

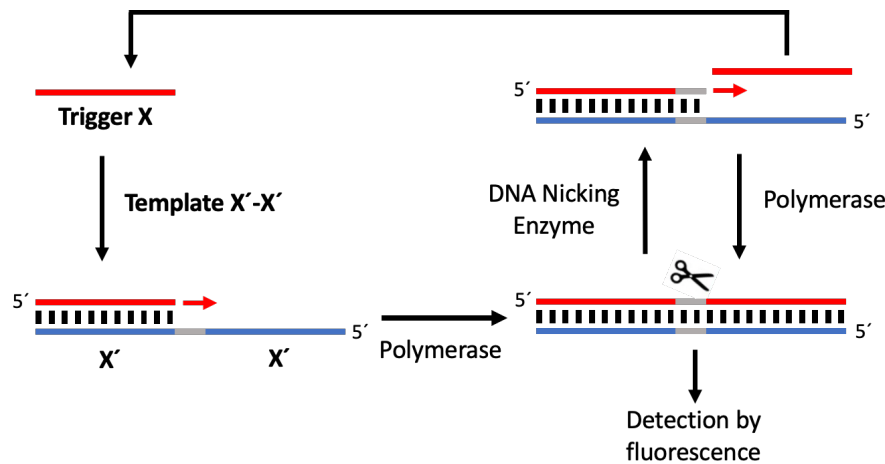
69 In the standard RT-PCR COVID-19 assay, reverse transcriptase converts the RNA of SARS-CoV-2
70 into cDNA prior to amplification (*vide supra*). The speed of this initial polymerisation reaction is a
71 significant limitation for this or potentially any other RNA detection method that proceeds via cDNA
72 amplification, including LAMP or EXPAR. We hypothesised that a faster method could be achieved
73 by generating a short DNA trigger sequence directly from the RNA genomic strand, without the need
74 for a lengthy reverse transcriptase step. Murray *et al* had previously demonstrated that the restriction
75 enzyme *Bst*NI could act as a nicking enzyme by selectively cleaving DNA within RNA:DNA
76 heteroduplexes.¹⁵ We considered that this enzyme could be used to generate the desired DNA
77 fragment for triggering the EXPAR reaction. To achieve this, we designed a 30-mer oligonucleotide
78 (called **Binder DNA**, Table 1) possessing a 5-base recognition site for *Bst*NI, as well as two partially
79 overlapping sequence stretches complementary to part of *Orflab* in the SARS-CoV-2 RNA genome
80 and the EXPAR DNA template (**Template X'-X'**). Site-selective cleavage of **Binder DNA** using
81 *Bst*NI would only occur in the presence of the RNA target from SARS-CoV-2, generating a shorter
82 strand of DNA, **Trigger X** (Scheme 1b). This shorter strand would now release from the heteroduplex
83 and bind preferably to the DNA template, as it can still form a fully-complementary 17-mer duplex
84 with the latter. Binding to the template would trigger EXPAR, with the newly released RNA strand
85 able to bind more **Binder DNA** to generate more **Trigger X**.
86

87 Applying this novel EXPAR approach in a two-stage process, we first undertook an enzymatic
88 digestion at 50 °C for five minutes of **Binder DNA** (1 μ M) in the presence of a sample of patient
89 SARS-CoV-2 RNA (72.7 copies/ μ L)¹⁶ obtained from PHE, before adding this solution to the EXPAR
90 reagent mix for the amplification step. This stage, performed in triplicate, gave an amplification time
91 of 3.17 ± 0.24 minutes, whereas no amplification was observed for the negative sample within 10
92 minutes (Fig. 1 and Supplementary Figure 3). To increase the speed of the RTF-EXPAR assay further,
93 we next investigated a “one-pot” approach by introducing *Bst*NI and **Binder DNA** to the EXPAR
94 reagents at the same time, before incubating and amplifying simultaneously at 50 °C. These assay
95 conditions gave an amplification time of only 4.00 ± 0.72 minutes for the positive sample, halving
96 the total assay time compared to the “two-pot” method (Fig. 1 and Supplementary Figure 4). Once
97 again, no signal change for the negative sample was observed within 10 minutes. As expected, this
98 was also the case for control experiments on the positive RNA sample in the absence of either **Binder**
99 **DNA** or **Template X'-X'** (see Supplementary Figures 5 and 6 respectively), and a sample of RNA
100 isolated from the CFPAC-1 human ductal pancreatic adenocarcinoma cell line (see Supplementary
101 Figure 7).
102

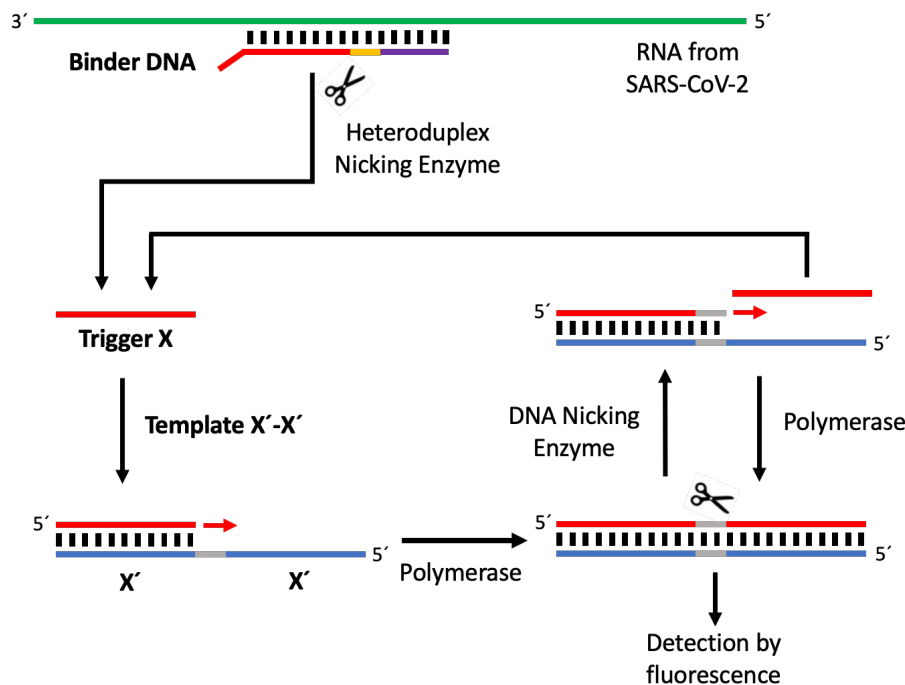
103 In conclusion, through the use of a new reverse transcriptase-free isothermal amplification method,
104 RTF-EXPAR, involving a DNA-selective restriction endonuclease, we have demonstrated the

105 successful detection of SARS-CoV-2 RNA in a total assay time of less than 5 minutes. This time is
 106 not only much faster than RT-PCR (assay time of at least 60 minutes) but also outperforms LAMP
 107 and 30-minute lateral flow antigen tests in current deployment. RTF-EXPAR would be completely
 108 compatible (i.e. deployment ready) for use on equipment currently used for RT-PCR COVID-19
 109 assays. Furthermore, the simplicity and speed of the assay enables this method to be modified to
 110 detect a range of infectious diseases caused by RNA-based pathogens (e.g. Ebola, RSV).

111
 112 (a) Exponential Amplification Reaction (EXPAR)

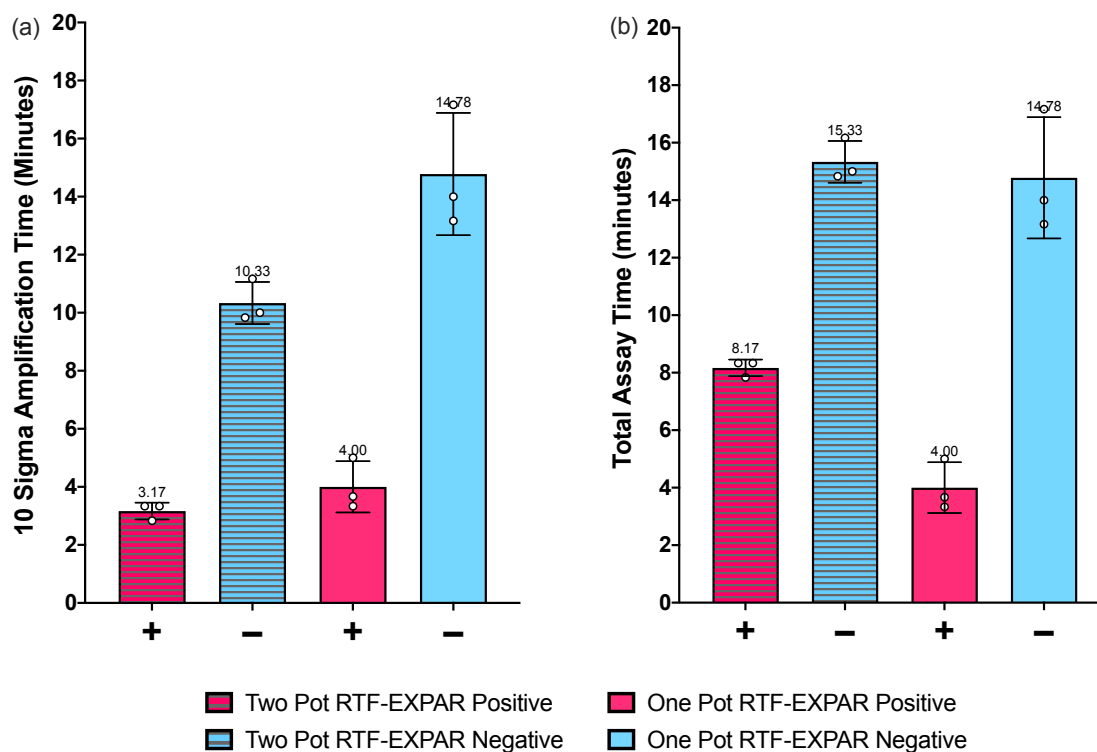


128
 129 (b) Reverse Transcriptase-Free EXPAR (RTF-EXPAR)



150 **Scheme 1.** (a) Reaction Scheme for EXPAR: **Trigger X** anneals to **Template X'-X'** and is extended by a
 151 DNA polymerase (*Bst* 2.0 polymerase); the top strand of the newly formed duplex DNA is then cut by a nicking
 152 enzyme (*Nt.Bst*NI); the released DNA (which is displaced by DNA polymerase in a subsequent extension
 153 reaction) is identical to **Trigger X** and is therefore able to prime another **Template X'-X'**. (b) Reaction Scheme
 154 for Reverse Transcriptase-Free EXPAR (RTF-EXPAR): **Binder DNA** anneals to viral RNA; the DNA strand
 155 of the DNA:RNA heteroduplex is cut by the restriction endonuclease *Bst*NI, which acts as a nicking enzyme
 156 by cutting the DNA strand only, the released DNA strand is **Trigger X**, which is then amplified by EXPAR.

157



158

159 **Figure 1.** RTF-EXPAR assay data for SARS-CoV-2 RNA detection (72.7 copies/ μ L, n = 3), showing: (a) the
 160 mean time for the amplification reaction only and (b) the mean total assay time from RNA sample to signal.
 161 Each run time was calculated to be the point at which the fluorescence signal was greater than 10 standard
 162 deviations from the baseline signal (10-sigma time). Error bars in datasets are the standard deviations of the
 163 10-sigma time. Signals observed for negative samples at >10 min are attributed to amplification arising from
 164 non-specific interactions.

165

166

167

168

Table 1. Oligonucleotides used in study.

Name	Sequence (5' – 3')
Trigger X	AGG GTA AAC CAA ATA CC
Trigger A	AGG GTT AAA CCA CCG CC
Trigger B	AGG GTC CTT AAC TTG CC
Trigger C	CCG GGA TTG GTT GAT
Template X'-X'	GGT ATT TGG TTT ACC CTG TGA GAC TCT GGT ATT TGG TTT ACC CT
Binder DNA	AGG GTA AAC CAA ATA CCT GG T GTA TAC GTT

169

170 Key: **Magenta** – Non-binding fragment, **Blue** – *Bst*NI recognition site (complementary to required
 171 5'-CCAGG-3' sequence in RNA target); **Bold** – **Trigger X** sequence in **Binder DNA** sequence

172

173

174

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180 interests.

181 182 Author Contributions

183 T.R.D, M.R.H and J.H.R.T supervised the project; J.G.C, J-L.H.A.D, L.O.I, T.R.D, M.R.H and
184 J.H.R.T designed the assay; A.D.B suggested the target gene and A.B. supplied the genomic material;
185 I.R.C and C.D.S designed the analysis software; J.G.C undertook the research; J.G.C, T.R.D and
186 J.H.R.T. wrote the manuscript.

187 188 Experimental Section

189 **Materials:** Milli-Q water purified with a Millipore Elix-Gradient A10 system (resistivity > 18
190 $\mu\Omega$.cm, TOC \leq 5ppb, Millipore, France) was used in all the experiments. Nt.*Bst*NBI, *Bst*NI and *Bst*
191 2.0 Polymerase were obtained from New England Biolabs (Hitchin, UK) as was the buffer, 10x
192 Isothermal amplification buffer (200 mM Tris-HCl, 100 mM (NH₄)₂SO₄, 500 mM KCl, 20 mM
193 MgSO₄, 1% Tween 20, pH 8.8) which was used in all the experiments. Superscript IV Reverse
194 Transcriptase was obtained from ThermoFisher (Paisley, UK), DMSO (> = 99%) was obtained from
195 Fisher Scientific (Loughborough, UK) and dsGreen 100x (an analogue of SYBR Green I), was
196 obtained from Lumiprobe (Hannover, De). Bovine Serum Albumin (BSA, diluted to 4 mg/mL in
197 water) and Single-Stranded Binding Protein (SSB, solution of 0.5 mgs in 20 mM Tris-HCl, pH 8.0,
198 0.5 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 50% Glycerol) was obtained from Sigma-Aldrich (Dorset,
199 UK). All the nucleotide triphosphates and oligonucleotide sequences (desalted) were obtained from
200 Sigma-Aldrich (Dorset, UK).

201
202 **PHE samples:** All clinical specimens were handled in a Containment Level 2 laboratory. To prepare
203 each sample, Viral Transfer Medium (VTM, 300 μ L, Medical Wire ViroCult) from a nose and throat
204 swab was added to Buffer AL (Qiagen) in a 1:1 ratio and heated to 60 °C for 30 minutes in a calibrated
205 heat block. Samples were then extracted on the MagNAPure96 (Roche) automated extraction system
206 and then run on the Abbott M2000 RT-qPCR Test for SARS-CoV-2 RNA Detection. For EXPAR
207 assay development, positive and negative samples from the SARS-CoV-2 RNA assays were
208 separately combined in MagNA Pure elution buffer (giving 29,080 RNA copies/ μ L for the combined
209 positive sample). Upon receipt from PHE, each sample was diluted 400-fold with water, aliquoted
210 into 50 μ L vials and stored at -80 °C. Prior to use, each sample was submerged in ice and allowed to
211 slowly melt; once melted the sample was used immediately before being cooled again for storage at
212 -80 °C.

213
214 **Data Analysis and classification:** To analyse the EXPAR real-time fluorescence amplification
215 curves and data, a program in C# was developed. The program analyses the first 10 data points and
216 calculates the mean value and standard deviation as a base line. Following generation of these two
217 values, each subsequent data point is analysed to determine if its value minus the average value is
218 greater than 10 standard deviations away from the mean. The cycle which meets this criterion is
219 converted into a time and used as the minimum amplification time. Under the concentrations and
220 conditions used in the RTF-EXPAR assay protocol, should the amplification time be less than 10
221 minutes, the output indicates the presence of SARS-CoV-2 RNA (true positive). For amplification
222 times greater than 10 minutes, the output indicates a complete test and the absence of SARS-CoV-2
223 RNA (false positive).

224 **RTF-EXPAR Assay Protocol:** The protocol first involves the preparation of three solutions, Part A,
225 Part B and Part C, followed by an addition step and then finally an amplification step.

226

227 *Part A:*

228 1.50 μL of water, 2.50 μL of 10x Isothermal amplification buffer, 3.75 μL of BSA solution,
229 1.50 μL of *Bst* 2.0 DNA polymerase (1.6 U/ μL), 0.75 μL of Nt.*Bst*NI (10 U/ μL).

230

231 *Part B:*

232 6.30 μL of water, 5.00 μL of 10x Isothermal amplification buffer, 0.75 μL of **Template X'-**
233 **X'** (1 μM), 2.40 μL of MgSO_4 (100 mM), 1.50 μL dNTP (10 nM), 0.75 μL of dsGreen (1:5
234 dilution in DMSO from 100x to 20x), 0.30 μL of SSB solution.

235

236 *Part C:*

237 (1) Sensitivity test (no RNA target): 3 μL of one trigger at **Trigger X** (100 nM, 10 nM, 1 nM, 100
238 pM, 10 pM, 1 pM and a blank)

239 OR

240 (2) Specificity test (no RNA target): 3 μL of one trigger at 100 nM (**Trigger X** or **Trigger A** or
241 **Trigger B** or **Trigger C**)

242 OR

243 (3) Reverse Transcriptase-Free EXPAR assay (two-pot RTF-EXPAR): 10 μL of RNA:DNA
244 heteroduplex digestion mixture, prepared as follows: 25 μL of water, 5 μL of 10x Isothermal
245 amplification buffer, 5 μL *Bst*NI (10 U/ μL), 10 μL of **Binder DNA** (1 μM) and 5 μL of viral sample.
246 The mixture is then incubated at 50 °C for 5 minutes.

247 OR

248 (4) Reverse Transcriptase-Free EXPAR assay (one-pot RTF-EXPAR): reagents are mixed together
249 in the following order: 1 μL *Bst*NI (10 U/ μL), 2 μL of **Binder DNA** (1 μM) and 3 μL of viral sample.

250

251 *Addition step:* Part B (17 μL) is added to a PCR tube, and to this is added Part C, followed by
252 Part A (10 μL). The tube is then sealed, with the contents then subjected to amplification.

253

254 *Amplification step:* Isothermal incubation and fluorescence signal measurements are performed using
255 an Agilent Mx3005P Real-Time PCR system (Didcot, UK) set to a constant temperature of 50 °C.
256 The fluorescence is measured every 10 seconds over an incubation time of 30 minutes.

257

258

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