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Reverse transcriptase-free detection of viral RNA using Hemo Klentaq DNA polymerase

Assol R. Sakhabutdinova, Rashit R. Gazizov, Alexey V. Chemeris, Ravil R. Garafutdinov *

Institute of Biochemistry and Genetics, Ufa Federal Research Center, Russian Academy of Sciences, 450054, Prosp. Oktyabrya, 71, Ufa, Bashkortostan, Russia

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ABSTRACT

COVID-19 pandemic highlighted the demand for the fast and reliable detection of viral RNA. Although various methods for RNA amplification and detection have been proposed, some limitations, including those caused by reverse transcription (RT), need to be overcome. Here, we report on the direct detection of specific RNA by conventional polymerase chain reaction (PCR) requiring no prior RT step. It was found that Hemo KlenTaq (HKTaq), which is posed as DNA-dependent DNA polymerase, possesses reverse transcriptase activity and provides reproducible amplification of RNA targets with an efficiency comparable to common RT-PCR. Using nasopharyngeal swab extracts from COVID-19-positive patients, the high reliability of SARS-CoV-2 detection based on HKTaq was demonstrated. The most accurate detection of specific targets are provided by nearby primers, which allow to determine RNA in solutions affected to multiple freeze-thaw cycles. HKTaq can be used for elaboration of simplified amplification techniques intended for the analysis of any specific RNA and requiring only one DNA polymerase.

1. Introduction

Viral diseases are a public health burden, accounting for more than half of infectious diseases worldwide [1]. A lot of severe diseases are caused by RNA viruses, such as influenza, AIDS, hepatitis A, C, D, E, and G, measles, poliomyelitis, Ebola haemorrhagic fever, etc. Recently, SARS-CoV-2 coronavirus has become a challenge caused the global COVID-19 pandemic and highlighted the need for the development of faster and more sensitive methods for detection of viral pathogens [2,3]. The demand for detection of SARS-CoV-2 RNA led to rapid elaboration of new advanced approaches based on isothermal amplification, microfluidics, CRISPR, biosensors, and other analytical techniques [4–10]. Nevertheless, polymerase chain reaction (PCR) still remains the gold standard and the most commonly used method for molecular diagnostics of infectious diseases [3,11].

Unlike DNA, two bottlenecks must be taken into account when RNA targets need to be detected. The first problem lies in low stability of RNA molecules, and special rules must be followed when handling with RNA-containing samples. The second one is due to the need for reverse transcription (RT) before PCR amplification, which requires the use of RNA-dependent DNA polymerase. The abovementioned issues complicate RNA analysis and often lead to false negative results [12–16]. It has been shown that the reliability of SARS-CoV-2 detection depends on the source of RNA [17,18], that forces the sampling of biomaterial with a higher viral load [19–22]. Various methods to improve the specificity and sensitivity of SARS-CoV-2 detection have been proposed as well [23–26].

However, it would be convenient to carry out RNA analysis using conventional PCR assays (i.e., without RT step) only with single enzyme which possesses both DNA polymerase and reverse transcriptase activities. For many years, such enzymes have been searched for, including the development of mutant forms [27,28], but only a few specific DNA/RNA-dependent DNA polymerases are commercially available, for example, RevTaq and RT-KTQ2 (https://www.mypols.de/). Some widely used DNA polymerases, such as Tth [29], Bst [30] and even Taq polymerase [31], are also known to possess reverse transcriptase activity under certain reaction conditions. For Taq polymerase, it was recently shown, that optimization of the buffer composition and thermal cycling protocol makes it possible to detect SARS-CoV-2 coronavirus RNA with high reliability [32]. This study aimed to demonstrate the applicability of Hemo KlenTaq DNA polymerase for direct detection of viral RNA using conventional PCR without RT step.

* Corresponding author.
E-mail addresses: sakhabutdinova.a.r@gmail.ru (A.R. Sakhabutdinova), rashit.r.gazizov@gmail.com (R.R. Gazizov), chemeris@anrb.ru (A.V. Chemeris), garafutdinovr@gmail.com (R.R. Garafutdinov).

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2. Materials and methods

2.1. Nucleic acids

The genetic material of SARS-CoV-2 coronavirus was obtained from nasopharyngeal swabs of the patients affected by COVID-19 (N = 50), using M-Sorb-OOM-96 extraction kit (Syntol, Russia). The COVID-19 diagnosis was confirmed by RT-PCR assay using RT-PCR-SARS-CoV-2 detection kit (Syntol, Russia). Nasopharyngeal swab extracts with SARS-CoV-2-uncertain (N = 25) or negative (N = 25) PCR results were used as well (Supplementary Table S1). The Rmix sample was prepared by mixing 20 RNA extracts of SARS-CoV-2 positive patients with a volume of 30 μl each. The resulting mix was divided into 5 aliquots followed by 2-, 5-, 10- or 20-fold freeze-thaw cycles at −20 °C, resulting in Rmix0 (no freezing), Rmix2, Rmix5, Rmix10 and Rmix20 samples, respectively.

Primers F1 (5′-ctagttatcagactcagactaattctcctc-3′), F2 (5′-gtttaataggggctgaacatgtcaacaac-3′) and R (5′-gactagctacactacgtgccc-3′) were designed using OligoAnalyzer (Integrated DNA Technologies) and purchased from Syntol (Russia). The nucleotide sequence of S-protein gene was chosen as an amplification target.

2.2. Isothermal amplification

All amplification samples were prepared in UVC/T-M-AR PCR box (Biosan). The working space, dispensers, and plasticware were preliminarily irradiated with ultraviolet for 20 min. Amplification was carried out in iQ5 thermal cycler (Bio-Rad Laboratories). Reaction mixtures with a volume of 20 μl contained 2.5 or 5 pmol of each primer, 1 or 2 μl of 2.5 mM dNTP, $1^{\times}$ DNA polymerase buffer, 0.1, 0.5 or $1^{\times}$ dsGreen intercalating dye (Lumiprobe, Russia), 0.2, 1.0 or 2.0 μl of Hemo KlenTaq DNA polymerase (New England Biolabs), or 2 a.u. of Synta-F Hot Start Taq **DNA polymerase** (SynGen, Russia). In some experiments, reaction mixtures contained 10 a.u. of Synta-RT (MMLV) reverse transcriptase and $0.5^{\times}$ Synta-RT buffer (SynGen, Russia). Test samples contained 1 μl of nasopharyngeal swab extracts from COVID-19 patients; non-template controls contained no RNA. Each sample was represented in two repeats. Three thermal cycling programs were used as follows. Program I: 1) RT step - 42°C for 10 min; 2) thermal cycling - 95°C for 10 s, 60°C for 20 s, 68°C for 5 s (>30 cycles). Program II: 1) thermal cycling - 95°C for 5 s, 60°C for 15 s, 68°C for 5 s (>30 cycles). Program III: 1) RT step - 42°C for 10 min; 2) thermal cycling - 85°C for 5 s, 60°C for 15 s, 68°C for 5 s (>30 cycles). All three programs contained five pre-cycles without fluorescent signal detection ('blind' cycles) which consisted of the following steps: 95°C for 10 s, 60°C for 20 s, and 68°C for 10 s.

3. Results and discussion

The most of the detection kits approved for molecular diagnostics of infectious diseases are based on amplification of DNA or RNA by polymerase chain reaction (PCR). For RNA targets, it is required to convert RNA into DNA copy before PCR using RNA-dependent DNA polymerases (reverse transcriptases). In experiments with mRNA, we observed that Hemo KlenTaq DNA polymerase (HKTaq) is likely able to provide cDNA synthesis using RNA molecules as templates (unpublished data). According to specification, HKTaq is intended for routine PCR and synthesizes DNA even in the presence of whole blood (https://international.neb.com/). Being a recombinant Taq polymerase, this enzyme could be expected to have reverse transcriptase activity, as was previously shown for wild-type Taq polymerase [31]. We aimed to evaluate the ability of HKTaq to synthesize DNA on RNA template and the applicability of HKTaq for the detection of viral RNA, using SARS-CoV-2 as an example.

It should be noted that low stability of RNA molecules imposes particular requirements on RNA isolation and storage. We believe that amplification failures arise due to RNA degradation can be avoided when nearby primers are used. Recently, the higher specificity and sensitivity of PCR with nearby primers as well as high reliability of degraded DNA amplification were demonstrated [33]. In this study, to evaluate the effect of primers disposition on efficiency of RNA amplification, two primer pairs were designed as shown in Fig. 1.

At the first step of the reaction, the R primer is annealed on a single RNA strand and extended by DNA polymerase with reverse transcriptase activity that results in corresponding cDNA. During the further PCR steps, primers F1 or F2 are annealed on the cDNA strand, and then ordinary PCR proceeds. The F1/R pair represents nearby primers and produces a short amplicon (54 bp), while F2 and R are conventional primers which lead to the 125 bp amplicon with regular size (i.e., > 100 bp). The proximity of the primers provides two advantages. Namely, in the case of weak reverse transcriptase activity, it is easier for DNA polymerase to move a short distance along the RNA template and form DNA amplicon with the size sufficient for further PCR. And for degraded RNA, it is more likely the short non-damaged targets available for amplification (amplifiable targets) will be kept.

It is considered that RNA-containing samples should be analyzed as soon as possible after biomaterial sampling, and cannot be stored for a long time and freeze to avoid RNA degradation and obtaining poor results. To study the specificity and sensitivity of HKTaq-based amplification of RNA, balanced RNA-containing samples were prepared by mixing nasopharyngeal extracts of patients affected by COVID-19. The Rmix0 sample (no freezing), and Rmix2, Rmix5, Rmix10 and Rmix20 samples, which were undergone to 2-, 5-, 10- or 20-fold freeze-thaw cycles were obtained, respectively.

Since there are no data on reverse transcriptase activity of HKTaq, three different thermal cycling programs were tested initially. Program I included the usual RT step at relatively low temperature (42 °C). Program II contained no incubation at 42 °C and included shortened denaturation (5 s), annealing (15 s), and elongation (5 s) steps. Recently, it was shown that even shorter durations of these steps (1, 5 and 1 s, respectively) are sufficient for the formation of short DNA amplicons with Taq DNA polymerase [34]. Program III contained an RT step and had reduced denaturation temperature (85 °C), which is suitable for efficient amplification with nearby primers as well. It turned out, that program II having the shortest duration (of about 20 min) provides the most efficient PCR. Thus, for HKTaq there is no need to add an RT step, and in subsequent experiments, program II was used only.

Then, optimization of reaction components was carried out by evaluation of their influence on the efficiency of amplification (Table 1). It turned out for F1/R pair, that the reduction of three key reaction components (HKTaq, primers and dNTP) accelerates amplification and increases specificity of PCR. This effect was clearly observed at decreased amount of HKTaq. Surprisingly, a 10-fold reduction of the enzyme amount resulted in a significant decrease of the threshold cycle (Ct) value (~6 cycles) for RNA-positive samples. A 2-fold decrease of the primers and dNTP concentration decreased Ct values as well, probably due to the suppression of nonspecific amplification caused by the competition between specific and nonspecific DNA synthesis [35].

Reduction of HKTaq, primers and dNTP amounts as well as addition of dithiothreitol resulted in the largest difference between Ct of the test samples and negative controls (~13 cycles) that provided a higher discrimination ability of the reaction. On the contrary, a decrease in the amount of reaction components slowed down amplification when using F2/R primers. Thus, reduced amounts of PCR components provided higher rate and specificity of the reaction when using only nearby primers; for further experiments, optimal concentrations of the reaction components were taken.

For primer pairs used, an unexpectedly considerable difference in the rate of the reaction was observed despite the same primers annealing temperature and amplification region. So, F1/R primers provided faster amplification (Ct (F2/R) - Ct (F1/R) > 10 cycles) compared to the F2/R pair (Fig. 2A, curves 1 and 4). Likely, the kinetics of RNA amplification with nearby primers differs from those one for conventional primers and requires a separate study.

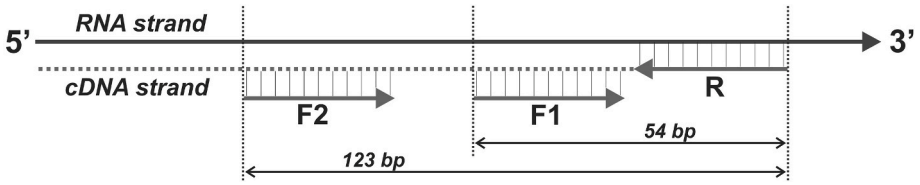

Fig. 1. Disposition of primers F1, F2 and R designed for RNA amplification.

Table 1
Average threshold cycle (Ct ± standard deviation) values depending on concentration of PCR components.

Reaction component	Final amount/ concentration		F1/R primer pair		F2/R primer pair	
			Rmix0	NTC[c]	Rmix0	NTC
HKTaq	standard/ recommended	1.0 µl[b]	21.3 ± 0.4	28.8 ± 0.9	27.5 ± 0.3	36.9 ± 1.7
primers		5 uM				
dNTP		250 uM				
dsGreen		1.0 ×				
dithiothreitol		0 M				
HKTaq	reduced[a]	0.1 µl[b]	15.6 ± 0.3	25.7 ± 1.7	29.3 ± 0.5	35.4 ± 1.8
		0.5 µl[b]	19.5 ± 0.5	27.1 ± 2.0	27.4 ± 0.4	35.8 ± 2.2
primers		2.5 uM	16.3 ± 0.6	25.8 ± 2.4	30.5 ± 0.6	35.3 ± 2.0
dNTP		125 uM	17.1 ± 0.5	24.9 ± 1.1	29.6 ± 0.5	35.4 ± 2.4
dsGreen		0.1 ×	18.4 ± 0.2	27.7 ± 0.9	26.3 ± 0.5	34.6 ± 2.1
		0.5 ×	20.1 ± 0.3	29.3 ± 1.6	27.1 ± 0.2	37.2 ± 2.3
dithiothreitol		10 uM	20.7 ± 0.4	27.1 ± 2.3	25.4 ± 0.6	37.8 ± 1.3
HKTaq	optimal	0.1 µl[b]	13.8 ± 0.3	26.4 ± 1.5	25.8 ± 0.5	37.0 ± 1.7
primers		2.5 uM				
dNTP		125 uM				
dsGreen		0.5 ×				
dithiothreitol		10 uM				

[a] Ct values for each reaction component at recommended amounts of other components.
[b] 1, 0.5 or 0.1 µl of HKTaq per 10 µl of the reaction.
[c] NTC - non-template control.

Nearby primers resulted in higher performance when degraded nucleic acids were amplified. So, Ct values, comparable to that for the initial Rmix0 sample, were obtained, even for the most degraded Rmix20 sample (Fig. 2A, curves 1 and 2). At the same time, for F2/R pair, the difference in Ct values for Rmix0 and Rmix20 reached ~9 cycles, indicating a significant decrease in amount of amplifiable RNA targets (Fig. 2A, curves 4 and 5). Melting curves analysis and gel-electrophoresis showed that the raise of fluorescence in non-template samples is due to non-specific products' formation, but not for primer dimers (Fig. 3C and D). Thus, disposition of the primers is decisive when degraded nucleic acids are analyzed, and for those one, the use of nearby primers is preferred.

It was observed that HKTaq provides amplification of specific targets with an efficiency similar to routine RT-PCR performed with MMLV reverse transcriptase and Taq DNA polymerase (Fig. 2B, curves 1, 2 and 3). Addition of 0.5 × MMLV buffer to the reaction mixture slightly impaired the efficiency of HKTaq-based amplification (Fig. 2B, curve 2), and the best result was obtained for HKTaq with native buffer only.

To determine the sensitivity of PCR with HKTaq, amplification was performed for diluted Rmix0 sample. Although, the quantity of RNA targets in Rmix0 was unknown, it was previously reported that nasopharyngeal swab extracts from COVID-19-positive individuals contain on average of about 10^4-10^5 target copies per 1 µl of solution, depending on the nature of biomaterial, viral load, RNA isolation technique and the method of samples handling [20–22]. It could be assumed that Rmix0 sample contained of about 10^4-10^5 target copies per 1 µl as well. Experiments with diluted Rmix0 sample (10-, 100-, and 1000-fold dilutions) showed that when using HKTaq and nearby primers, accurate detection of about 10^2-10^3 copies is achieved (Fig. 3A). Such a limit of detection indicates high sensitivity and correlates with the results obtained earlier for quantitative RT-PCR assays performed without specific fluorogenic probes [13–15].

The experiments with multiple-frozen RNA samples showed, in general, a decrease in the number of amplifiable RNA targets for both primer pairs used (Fig. 3B).

However, for nearby primers, Ct values decreased by only 2–3 cycles that corresponds to less than 10-fold decrease in the amount of amplifiable targets; and even for Rmix20 sample, ΔCt exceeded 5 cycles that makes it possible to detect specific RNA with high reliability. As for F2/R primers, ΔCt values dropped by 5 cycles even for Rmix5 sample, that indicates the low copy number of amplifiable targets, and detection can be considered unreliable in this case. Thus, the proximity of the primers allows to detect degraded nucleic acids that enable to impose gentle requirements for transportation and storage of RNA-containing materials.

4. Conclusion

Detection of any specific RNA has been in great demand in recent decades, given the role of RNA in pathogenesis and development of different diseases. For RNA targets, it would be convenient to exploit PCR assay without common RT step, that requires polymerases able to synthesize DNA both on DNA and RNA templates. It was previously reported that widely used Taq DNA polymerase has reverse transcriptase activity. However, researchers avoid to use this enzyme for RNA quantification due to the low reliability and reproducibility of the results obtained, which strongly depend on the source of the enzyme (i.e., depend on the manufacturer and production batch) and reaction conditions. We found that specific Hemo KlenTaq DNA polymerase, offered as DNA-directed DNA polymerase and used for routine PCR, provided efficient amplification taking RNA as an initial template for DNA synthesis. Using RNA-containing extracts from COVID-19 patients, it was shown that HKTaq allows to detect RNA targets with high sensitivity and specificity. HKTaq allows immediate PCR cycling, i.e., does not require a reverse transcription step. The use of HKTaq along with nearby primers strongly accelerates amplification and reduces duration of the reaction compared to conventional primers. When nearby primers are used, it becomes possible to detect RNA with high accuracy even in samples affected by multiple freezing. It was shown that the decrease in the amount of some reaction components, especially HKTaq, increases the reliability of PCR. It can be assumed that other specific Taq polymerases would be suitable for quantitative PCR amplification of RNA, e.g., for genes expression analysis, long non-coding RNA studies or other

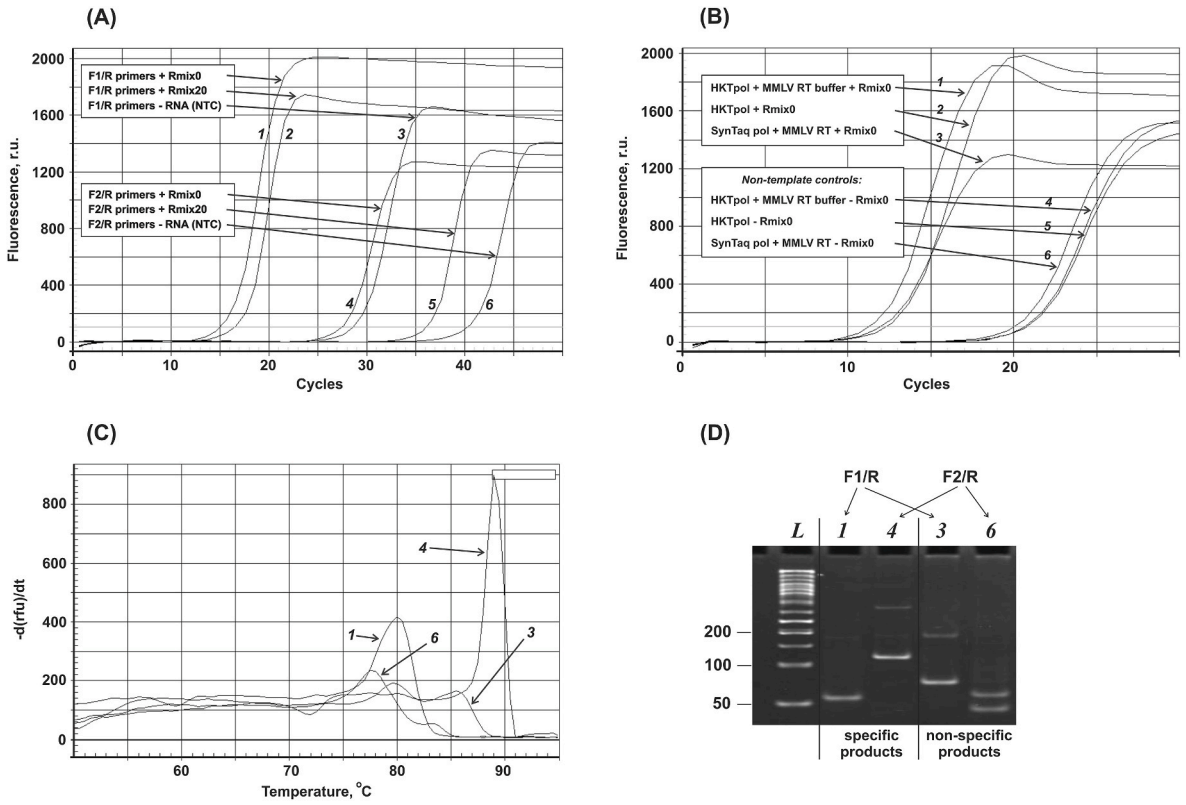

Fig. 2. Real-time PCR amplification of SARS-CoV-2 coronavirus RNA. (A) The difference in amplification rate for nearby (F1/R) and conventional (F2/R) primers; (B) Amplification efficiency for HKTaq and Taq pol + MMLV RT; (C) melting curves analysis for F1/R (curves 1 and 3) and F2/R (curves 4 and 6) primer pairs; (D) formation of specific (lanes 1 and 4) and non-specific (lanes 3 and 6) amplification products.

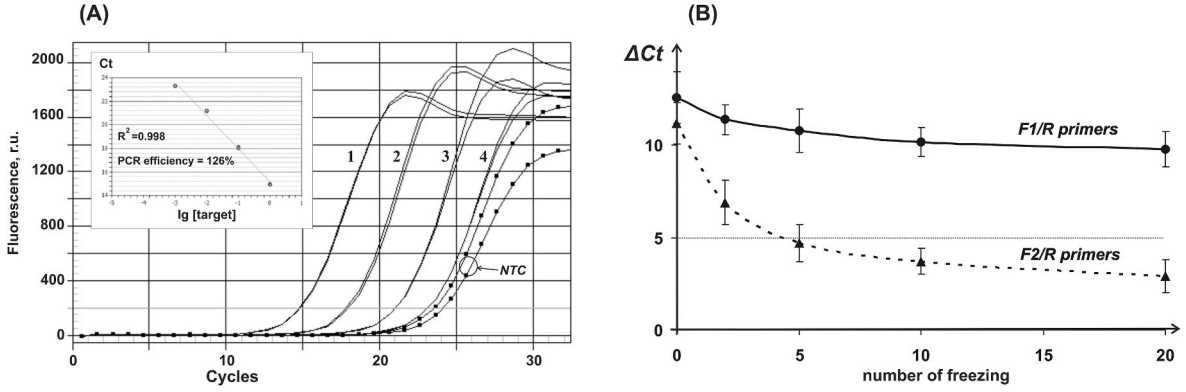

Fig. 3. Analytical performance of HKTaq-based amplification of RNA. (A) Amplification of diluted Rmix0 sample. Curves: 1 - Rmix0 sample without dilution, 2 - 10-fold dilution of Rmix0, 3 - 100-fold dilution of Rmix0, 4 - 1000-fold dilution of Rmix0. B) The influence of multiple freezing of RNA solution on Ct values obtained for nearby F1/R (solid line) and conventional F2/R (dashed line) primers. ΔCt values were determined as follows: ΔCt = Ct(NTC) - Ct(RmixN), NTC - non-template control. The line at ΔCt = 5 denotes the threshold of analytical significance.

applications.

CRediT authorship contribution statement

Assol R. Sakhabutdinova: Investigation, Formal analysis, Validation, Writing – original draft. **Rashit R. Gazizov:** Investigation, Visualization. **Alexey V. Chemeris:** Conceptualization, Methodology. **Ravil R. Garafutdinov:** Conceptualization, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114960.

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