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Producing ready-to-use qPCR for detection of DNA from Trypanosoma cruzi or other pathogenic organisms

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TITLE:

Ready-To-Use qPCR for Detection of DNA from *Trypanosoma cruzi* or Other Pathogenic Organisms

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KEYWORDS:

ready-to-use, PCR, diagnostics, *Trypanosoma*, laboratory routine, neglected diseases, tropical diseases

SUMMARY:

The present work describes the steps for producing ready-to-use qPCR for *T. cruzi* DNA detection that can be pre-loaded on the reaction vessel and stored in the refrigerator for several months.

ABSTRACT:

Real-time PCR (qPCR) is a remarkably sensitive and precise technique, which allows for the amplification of minute amounts of nucleic acid targets from a multitude of samples. It has been extensively used in many research areas and achieved industrial application in fields such as human diagnostics and trait selection in crops of genetically modified organisms (GMO) crops. However, qPCR is not an error-proof technique. Mixing all reagents into a single master mix that is subsequently distributed onto 96 wells of a regular qPCR plate might lead to operator mistakes such as incorrect mixing of reagents or inaccurate dispensing into the wells. Here, a technique called gelification is presented, whereby most of the water present in the master mix is substituted by reagents that form a sol-gel mixture when submitted to a vacuum. As a result, qPCR reagents are effectively preserved for a few weeks at room temperature or a few months at 2–8 ^{o}C. Details of preparing each solution are shown here along with the expected aspect of a

gelified reaction designed for the detection of *T. cruzi* satellite DNA (satDNA). A similar procedure can be applied for the detection of other organisms. Starting a gelified qPCR run is as simple as removing the plate from the refrigerator, adding the samples to their respective wells, and starting the run, thus decreasing the setup time of a full-plate reaction to the time it takes to load the samples. Additionally, gelified PCR reactions can be produced and controlled for quality in batches, saving time and avoiding common operator mistakes while running routine PCR reactions.

INTRODUCTION:

Chagas disease was discovered in the early 20th century in rural regions of Brazil, where poverty was widespread[1,2]. Even today, the disease continues to be connected to social and economic determinants of health in the Americas. Chagas disease is biphasic, comprising an acute and a chronic phase. It is caused by infection by the *Trypanosoma cruzi* parasite, being transmitted by insect vectors, blood transfusions from mother to child during pregnancy, or oral ingestion of contaminated food[3,4].

The diagnostic of Chagas disease can be made through the observation of clinical symptoms (especially the Romaña sign), blood smear microscopy, serology, and molecular tests such as real-time PCR (qPCR) or isothermal amplification[4–9]. Clinical symptoms and blood smear microscopy are used in suspected cases of acute infections, while the search for antibodies is used as a screening tool in asymptomatic patients. Because of its sensitivity and specificity, qPCR has been suggested to be used as a monitoring tool for chronic patients, for acute patients undergoing treatment measuring the parasite load in the blood, and as a surrogate marker of therapeutic failure[6,8,10–12]. Although more sensitive and specific than currently available tests, qPCR is effectively prevented from being known as diagnostic tools in underprivileged regions worldwide due to the requirement of freezing temperatures for transportation and storage[13–15].

To circumvent this obstacle, conservation techniques such as lyophilization and gelification have been explored[16,17]. While lyophilization provides conservation for years, it requires specially made reagents without the presence of glycerol, which is commonly used for enzyme stabilization/conservation[18]. While gelification has been shown to provide conservation for months, it allows the use of regular reagents[19]. The gelification solution comprises four components, each with specific roles in the process: the sugars trehalose and melezitose protect the biomolecules during the desiccation process by reducing free water molecules in the solution, glycogen produces a broader protective matrix, and the amino acid lysine is used as a free radical scavenger to inhibit the oxidizing reactions between the biomolecule's carboxyl, amino and phosphate groups. These components define a sol-gel mixture that prevents the loss of the tertiary or quaternary structure during the desiccation process, thus helping to maintain the biomolecules' activity upon rehydration[19]. Once stabilized inside the reaction tubes, the reactions can be stored for a few months at 2–8 °C or a few weeks at 21–23 °C instead of the regular -20 °C. This approach has already been incorporated in tests designed to help diagnose diseases such as Chagas disease, malaria, leishmaniasis, tuberculosis, and cyclosporiasis[13–15,20].

The present work describes all the steps to prepare the required solutions for the gelification

procedure, the pitfalls in the process, and the expected final aspect of a ready-to-use gelified qPCR inside eight-tube strips. The same protocol can be adapted for single tubes or 96-well plates. Finally, the detection of *T. cruzi* DNA will be shown as a control run.

PROTOCOL:

1. Preparation of stock solutions and gelification mixture

NOTE: Four stock solutions will be prepared (400 mg/mL of melezitose, 400 mg/mL of trehalose, 1.5 mg/mL of lysine, and 200 mg/mL of glycogen) and mixed according to the proportion shown in **Table 1** to produce the gelification mixture. Although the protocol describes 10 mL of stock solutions production, it can be adapted for lower or higher volumes.

1.1. Melezitose solution

1.1.1. Weigh 4 g of melezitose in a 15 mL plastic tube, add 6 mL of nuclease-free water, and vortex at the maximum speed of the instrument until the powder is solubilized.

NOTE: More water can be added to facilitate solubilization, taking care not to exceed the final volume (see below).

1.1.2. Make up the final volume to 10 mL with nuclease-free water. Label and store at 2–8 °C for up to 6 months.

1.2. Trehalose solution

1.2.1. Weigh 4 g of trehalose in a 15 mL plastic tube, add 6 mL of nuclease-free water, and vortex at the maximum speed of the instrument until the powder is solubilized.

NOTE: More water can be added to facilitate solubilization, taking care not to exceed the final volume (see below).

1.2.2. Make up the volume to 10 mL with nuclease-free water and filter the solution through a 0.2 µm filter. Label and store at 2–8 °C for up to 6 months.

1.3. Glycogen solution

1.3.1. Weigh 2 g of glycogen in a 15 mL plastic tube, add 6 mL of nuclease-free water, and vortex at the maximum speed of the instrument until the powder is solubilized.

NOTE: More water can be added to facilitate solubilization, taking care not to exceed the final volume (see below).

1.3.2. Keep the solution at rest at 2–8 ˚C for 8–12 h because the solubilization of glycogen produces lots of bubbles (**Figure 1**). Make up the volume to 10 mL with nuclease-free water. Label and store at 2–8 ˚C for up to 6 months.

1.4. Lysine solution

1.4.1. Weigh 7.5 mg of lysine in a 15 mL plastic tube, add 6 mL of nuclease-free water. Vortex at the maximum speed of the instrument until the powder is solubilized.

NOTE: More water can be added to facilitate solubilization, taking care to not exceed the final volume (see below).

1.4.2. Make up the volume to 10 mL with nuclease-free water and filter the solution through a 0.2 µm filter. Transfer the solution to an amber flask or protect it from light. Label and store at 2–8 ˚C degrees for up to 6 months.

1.5. Gelification Mixture (GM)

1.5.1. In a 50 mL plastic tube, mix the volumes of stock solutions according to **Table 1**.

1.5.2. Mix the reagents by ten end-to-end inversions of the tube.

NOTE: If this step is performed in a laminar flow safety hood, there is no need for a filtration step. If this step is not performed in a clean environment, filter the solution through a 0.2 µm filter before transferring it to an amber flask.

1.5.3. Transfer the solution to an amber flask or protect it from light. Label and store at 2–8 ˚C for up to 3 months.

NOTE: As a quality control step for preparing the gelification mixture, ensure that the measured pH, conductivity, and density values are within the following ranges: pH 5.55–6.66; conductivity 0.630–0.757 mS/cm; and density 1.08–1.11 g/cm^3. All measurements should be taken at 25 ˚C.

2. Preparation of qPCR master mix for gelification

NOTE: In this step, the qPCR master mix for gelification is prepared. Hence, water is not added to the mix but instead, the gelification mixture is added (**Table 2**).

2.1. Thaw the reagents in a refrigerated container. Mix the reagents in a 1.5 mL tube according to **Table 2**. An example of a reaction with a final volume of 25 µL containing 5 µL of DNA sample is shown here.

NOTE: The DNA sample is not added to the mixture in this step; it is used here solely to calculate the final volumes of each reagent of the qPCR master mix. DNA samples should be added right before starting the run (see step 4 below).

3. Gelification of the reagents on the reaction vessels

3.1. Appropriately multiply the volumes shown in **Table 2** for preparation of an eight-tube strip or a 96-well plate.

3.2. Pipet 18.5 µL of the gelification master mix shown in **Table 2** onto each reaction well.

NOTE: This volume represents the volumes of oligonucleotide mix, PCR buffer, and gelification mixture used for one reaction (according to **Table 2**) and will vary depending on the concentration of the reagents and the volume needed for one reaction. The final volume in the gelification master mix is different from the volume in a regular master mix because water is not added.

3.3. Place the tubes/plate in the heat-conductive support (e.g., aluminum) inside the vacuum oven.

NOTE: The heat-conductive support is optional. The operator must ensure that the bottom of the tubes is in contact with the vacuum oven shelf to allow fast thermal equilibrium.

3.4. Place one bentonite clay bag for every two 96-well plates.

NOTE: Bentonite clay bags are used for absorbing the water that is being removed from the gelification master mix by the differential pressure exerted by the vacuum. Bentonite clay bags were found to be unnecessary for less than two 96-well plates.

3.5. Subject the tubes/plate containing the gelification master mix to three vacuum cycles (30 ± 5 mBar) of 30 min each, alternating with vacuum release until the atmospheric pressure is achieved (900–930 mBar), under controlled temperature (30 °C ± 1 °C) (**Figure 2**).

NOTE: The instrument uses software to control the parameters, and an example of the cycle is shown in **Figure 2**. The user must create the profile for the run, indicating the chosen parameters.

3.6. When the cycle is completed, check the tubes/plates for proper gelification of the reagents by ensuring that the volume is visibly reduced (**Figure 3**) and that the liquids do not move upon tapping the tubes/plates with fingers.

NOTE: If gelification did not occur, the solution would splatter on the tube walls when tubes are tapped (**Figure 3**).

3.7. Seal and store the tubes/plates at 2–8 °C for 8–12 h before use.

4. Using a gelified qPCR

4.1. Remove the tube strip or plate from the refrigerator and open it in a workstation for sample manipulation. Add 15 µL of nuclease-free water to each reaction vessel.

NOTE: The volume of gelified reagents is considered to be about 5 µL. So, together with the DNA sample volume (see below), the final reaction volume is 25 µL.

4.2. Add 5 µL of DNA sample.

NOTE: Any qPCR-quality DNA template might be used. In the present work, DNA was extracted from 10^8 *T. cruzi* epimastigotes (strain Dm28c) and was serially diluted at a 1:10 ratio using TE buffer.

4.3. Seal the tubes/plates and proceed to the equipment of choice. Run the experiment and proceed to regular data analysis.

REPRESENTATIVE RESULTS:

Three of the reagents that form the gelification mixture are easily solubilized upon vigorous vortexing. However, glycogen requires careful vortexing to ensure the powder has been completely solubilized. Unfortunately, vigorous vortexing produces lots of bubbles, which makes it difficult to determine the actual volume of the solution (**Figure 1A–B**). Therefore, it is essential to let the glycogen solution rest in the refrigerator until most of the solution trapped within the bubbles has moved down to the main solution body. Considering the production protocols and the lab routine, gelified plates are kept in the refrigerator overnight (or around 8–12 h), resulting in the settling of most of the bubbles, thus making it easier to determine the correct volume and adjust to the desired final volume (**Figure 1C**). Note the difference in the volume of bubbles between the glycogen tubes in **Figures 1B**,**C**, respectively, right after the solubilization and after overnight settling.

Once the gelification mixture is added to the qPCR master mix in substitution for water (**Table 2**), the tube strips or plates are ready to go to the vacuum oven. The shelves of the vacuum oven contain a Peltier heating element, ensuring that any tubes that are in contact with it remain at the same temperature. In the present protocol, the temperature inside the chamber is kept constant at 30 ˚C, while the pressure varies between 910–930 mBar (atmospheric pressure) and 30 mBar (near-vacuum). **Figure 2** shows these two variables plotted over time, showing the constant temperature (green line, upper panel) and the variation of pressure (red line, lower panel). After the cycles are finished, the master mix inside the well decreases in volume and becomes gelified at the bottom, i.e., without moving or splashing when the tubes are tapped with fingers (**Figure 3**). The tubes can now be capped and stored at 2–8 ˚C. The reactions will fail to gelify if the gelification mixture (**Table 1**) is incorrectly prepared; the proposed quality control step should see the fault before mixing the gelification mixture with qPCR reagents.

To be used, the gelified reagents inside the tubes/plates must be resuspended in nuclease-free water and the DNA sample diluted usually in water or TE buffer. The resuspension of the reagents of the sol-gel mixture is achieved during the first step of denaturation of the qPCR thermal protocol (usually, 5–10 min at 95 ˚C), so no extra step is required. **Figure 4A** shows representative traces of the qPCR detection of *T. cruzi* DNA using published oligonucleotide sequences[15]. Suboptimal results include loss of sensitivity, which can be tested with a dilution curve of a solution with a known concentration of genomic targets and loss of specificity, which can be tested with a panel of related trypanosomatid organisms. **Figure 4B** shows the loss of sensitivity that might arise when the gelification process is not correctly executed or when the reaction loses its stability after being stored at 2–8 ˚C for more than 6 months.

FIGURE AND TABLE LEGENDS:

Figure 1: Solubilization of glycogen produces lots of bubbles. Because glycogen produces too many bubbles during solubilization, the glycogen solution must be kept at rest before adjusting to the final volume. (**A**) Bubbles formed during vortexing. (**B**) All of the powder was solubilized, but it is not possible to determine the final volume because of the excess bubbles. (**C**) After 12 h in the refrigerator (tube in the middle), the volume of bubbles is reduced.

Figure 2: Vacuum cycling (lower panel) and temperature control (upper panel). Representative traces of temperature (upper panel) and pressure (lower panel) variation are shown. Black lines represent the programmed variations, whereas the green and red lines represent actual readings of the instrument.

Figure 3: Aspects of gelified master mix inside an eight-tube strip. (**A**) qPCR master mixes before the vacuum exposure. (**B**) Liquid splatters on the tube walls because of incomplete gelification (only one vacuum cycle). (**C**) Gelified qPCR reagents with a clear visible reduction in volume. The liquid does not splatter on the walls when the tubes are tapped.

Figure 4: Representative traces of gelified master mixes detecting DNA from *T. cruzi* epimastigotes. DNA extracted from *T. cruzi* epimastigotes (10^8 cells) was serially diluted at a 1:10 ratio, and the DNA concentrations ranging between 10^4 and 10^0 cells were subjected to detection using a gelified qPCR. (**A**) The expected result of correctly gelified qPCR (**B**) Detection of the same samples using a plate where the gelification was not properly executed, resulting in loss of sensitivity. Note that the lower concentrations are detected less frequently in panel **B**.

Table 1: Stock concentrations and volumes of solutions used to produce 20 mL of the gelification mixture. The volume of each stock solution must be proportionally adjusted to produce lower or higher final volumes of the gelification mixture.

Table 2: Volumes of reagents to produce qPCR master mixes for regular or gelified reactions. The difference between the two master mixes is that water is added to the regular master mix whereas the gelification mixture is added (i.e., instead of water) to the gelification master mix.

DISCUSSION:

Recent years have highlighted the need to find more sensitive and specific technologies to help diagnose tropical and neglected diseases. Although important for epidemiological control, parasitological (optical microscopy) and serological tests have limitations, especially regarding sensitivity and point-of-care applicability. DNA amplification techniques such as PCR, isothermal amplification, and respective variations have long been used in laboratory settings, but technological hurdles preclude it from being used in field settings. One of the main obstacles is the need for temperatures of -20 ˚C for transportation and storage of the reagents. To remediate this situation, techniques such as lyophilization and gelification have been used to store PCR reactions out of the freezer[16,18,19].

The present work shows all the steps necessary to gelify a qPCR reaction to detect *T. cruzi* DNA inside the reaction vessel, be it tubes, tube strips, microfluidic chips, or plates. Preliminary studies using an RT-LAMP reaction suggest that the gelification technique may also be used to preserve and shield other nucleic acid amplification and modification enzymes, as described by Rosado et al.[19]. Although relatively straightforward, the two steps that cause most of the operator mistakes in qPCR routines are (a) the preparation of glycogen and melezitose solutions and (b) calculation of the volume of the reaction mix to be added to each reaction tube before the vacuum step. First, the glycogen solution must be refrigerated overnight before the final volume adjustment, and the melezitose solution must be vigorously vortexed (possibly with mild heating at 50 ˚C) for complete solubilization. Second, the researcher planning the experiment must be aware that the reagents' volumes calculated pre-vacuum might be uneven since water is not added to round up to the reaction volume. The actual reaction volume will be obtained when the gelified reaction is re-solubilized by the addition of sample and water, before running the PCR.

The biggest limitation of the method is the stability of the reactions, which is around 6–8 months at 2–8 ˚C[14,15]; it is considerably shorter than lyophilized reactions, which may remain stable for years[18]. Depending on the specificity of the oligonucleotide sequences, unspecific binding and amplification might occur, which should be carefully examined by the researchers. For example, Costa and collaborators report that the annealing temperature of the gelified qPCR for detection of *C. cayetanensis* had to be adjusted in +1 ˚C to avoid unspecific amplifications[15,21]. Similarly, researchers should avoid using enzymes that might be regulated by or use the gelification components as substrates.

The gelification technique is particularly useful because of its ease of use in the laboratory routine as well as an introduction into a production line[16,19,22] allowing smooth quality control; the latter in turn enables robust and comparable data across multiple operators and effectively eliminates common operator mistakes at crucial steps, with a bonus of eliminating freezing temperature requirements during transportation and storage. Preliminary studies suggest that eliminating the cold chain would result in an overall reduction of cost by up to 20% for a qPCR test[14]. Elimination of the cold chain also makes it feasible to implement qPCR as a confirmatory test for neglected diseases such as Chagas disease in underdeveloped regions, thus favoring their epidemiological control[23].

Finally, the gelification protocol streamlines the use of qPCR tests as it only requires the user to

add water and the extracted *T. cruzi* DNA, avoiding errors during reagent handling, and decreasing the set-up time as well as the possibility of the reagent's contamination. Such characteristics provide efficiency for a routine diagnostic laboratory, speeding the delivery of results to patients and increasing the reliability of the diagnosis. Lastly, because it dispenses the need for a -20 ˚C cold chain, it is suitable for diagnostic use in low-resource environments.

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DISCLOSURES:

The authors declare no conflicts of interest.

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Figure 1

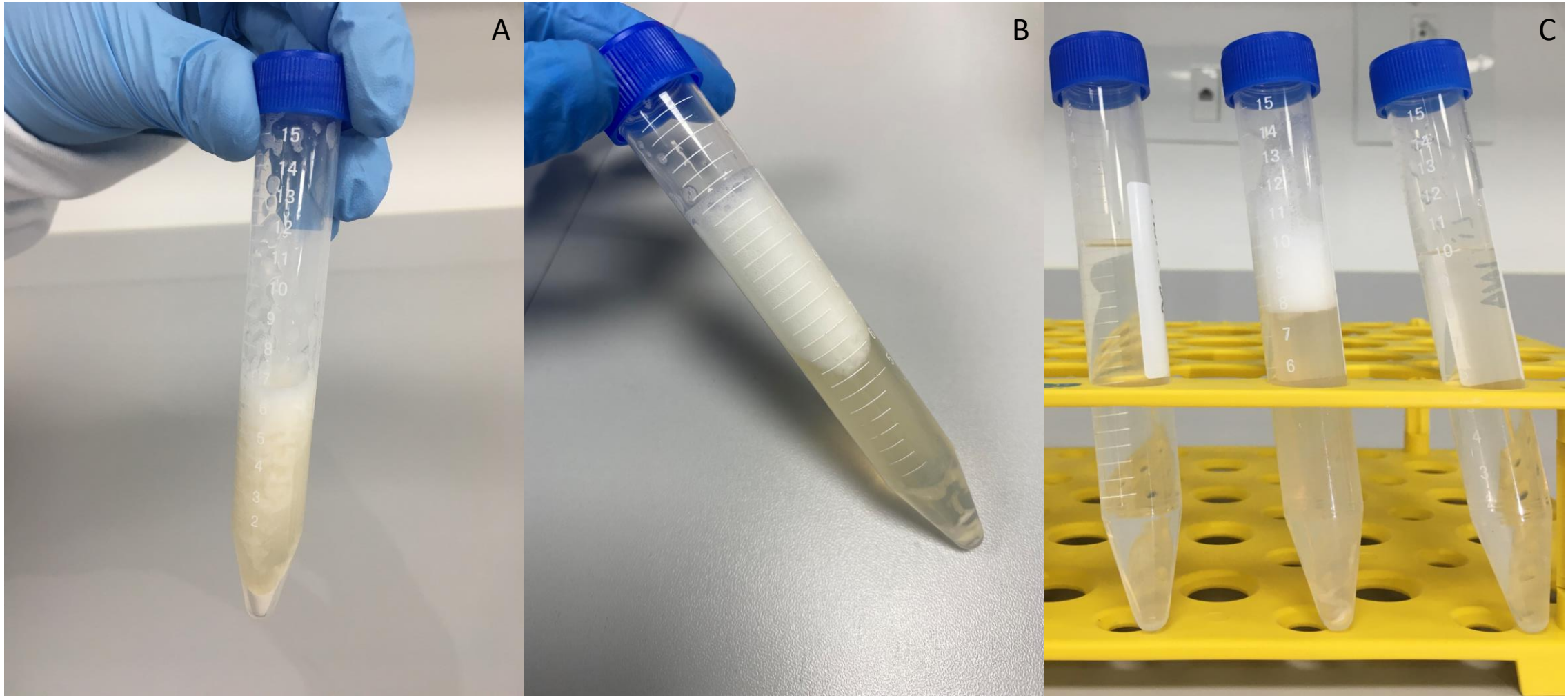


Figure 2

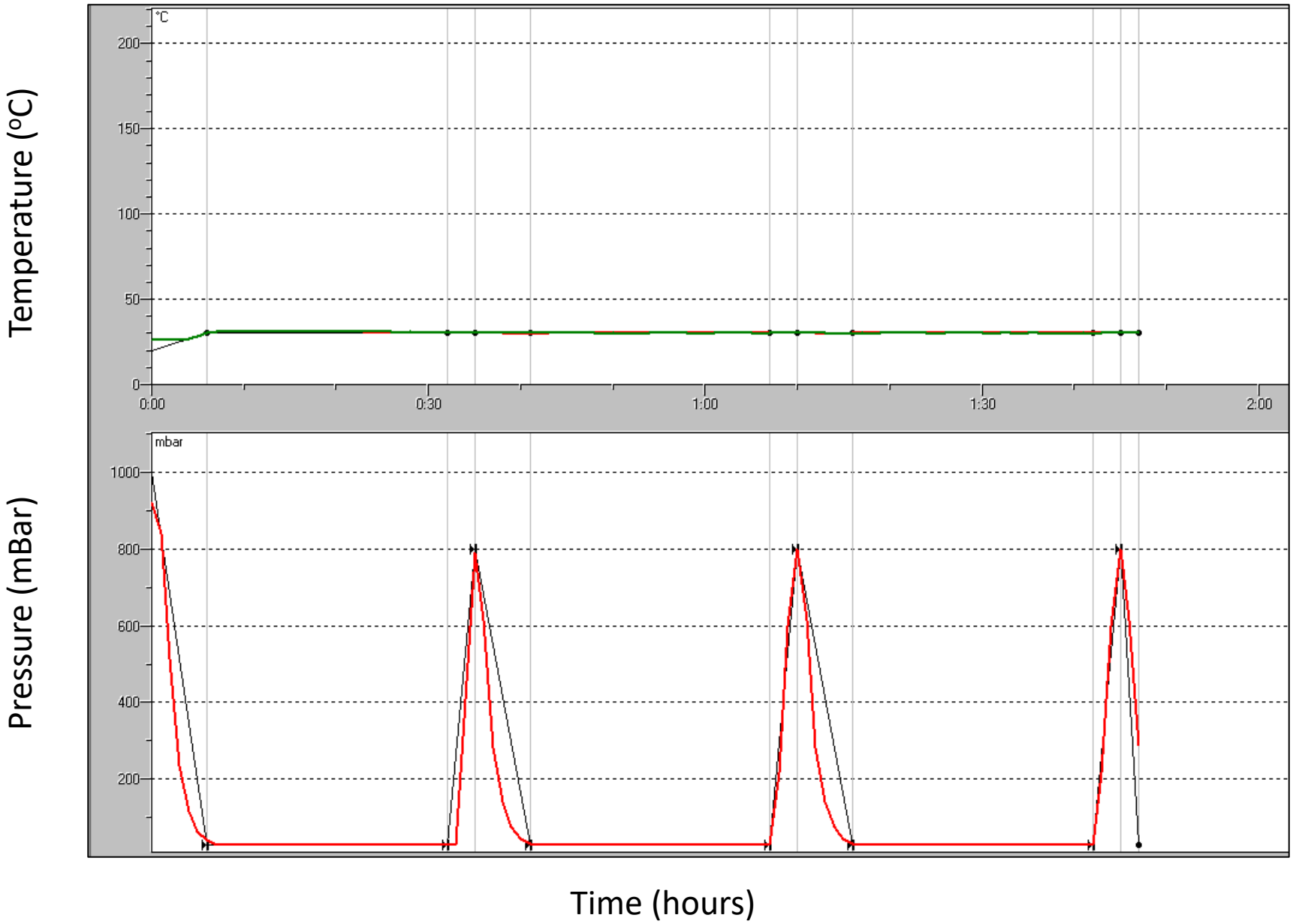


Figure 3

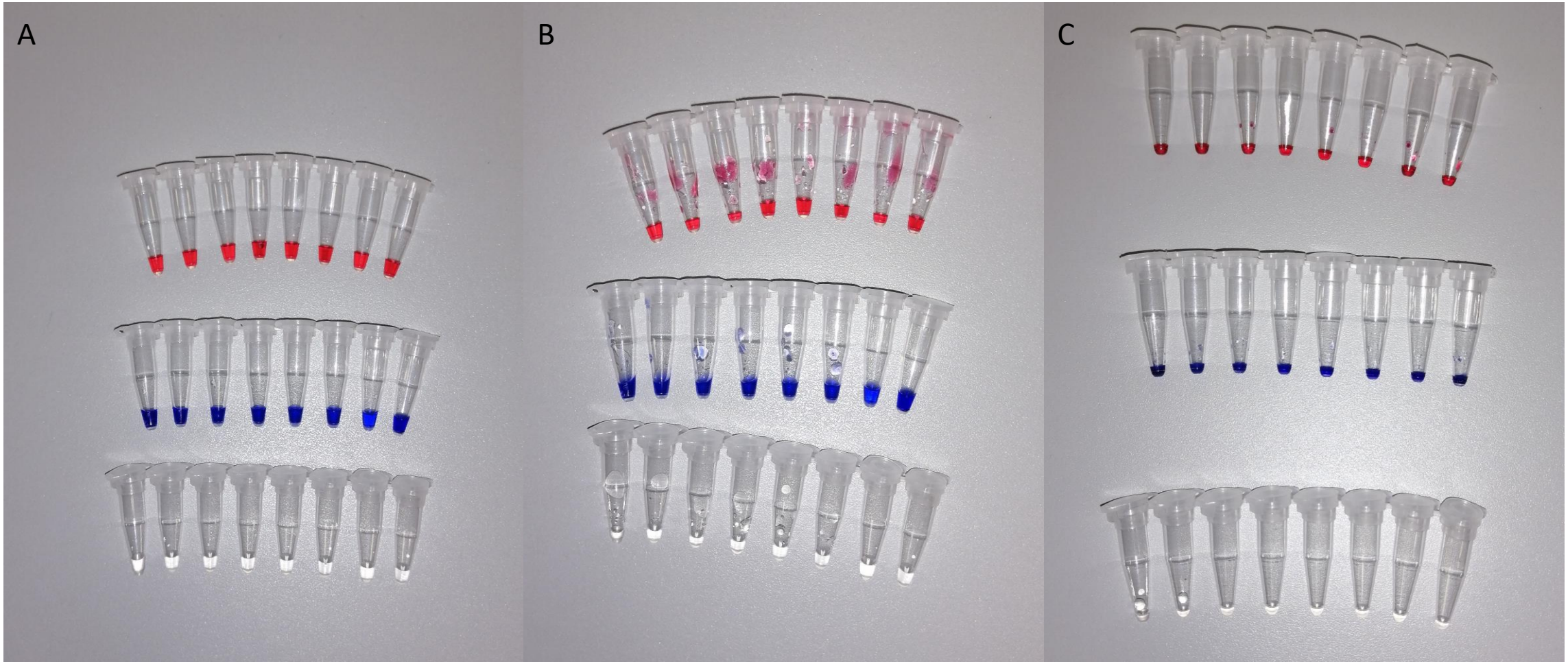
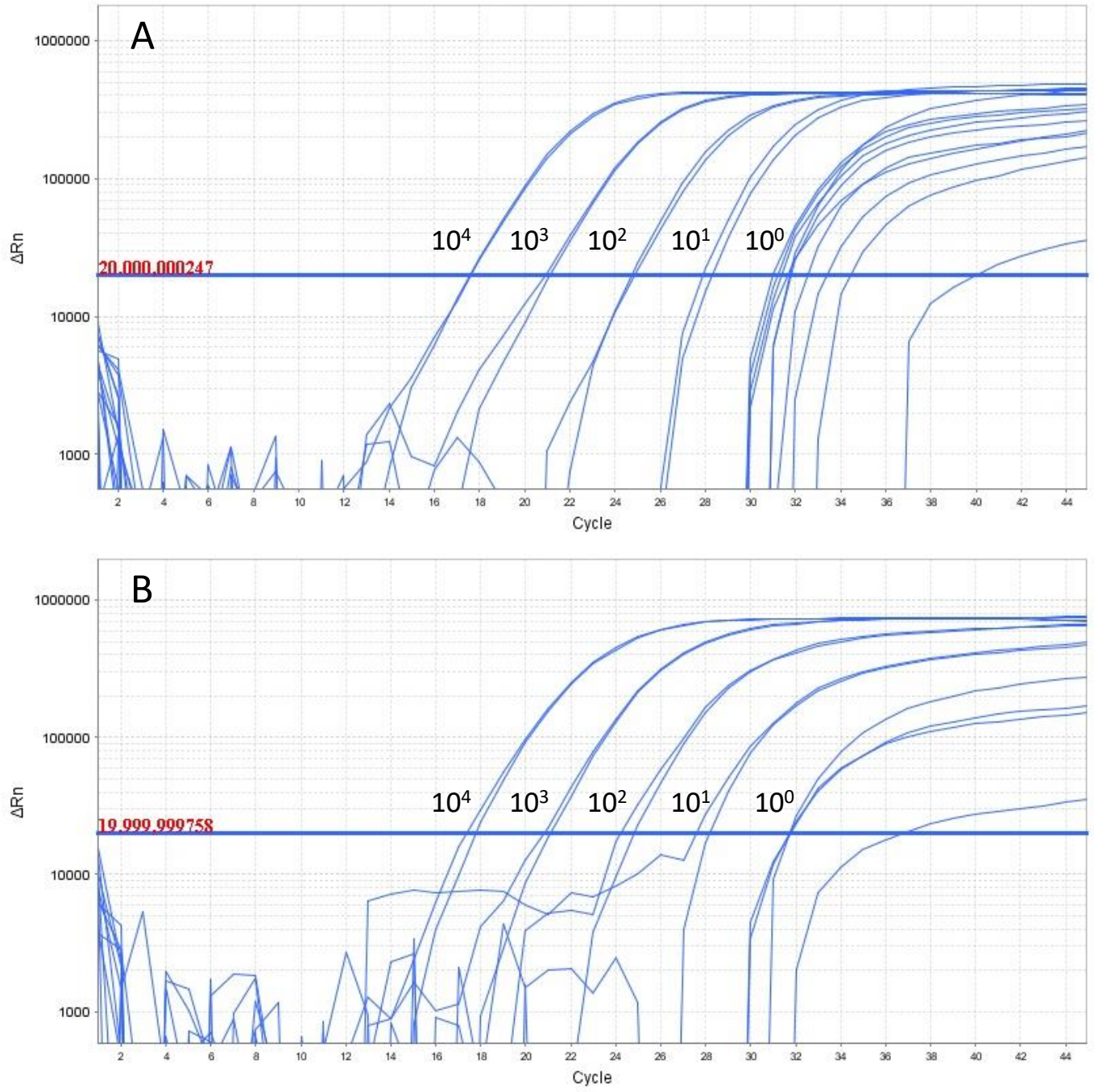


Figure 4



Table

Solution	Stock concentration	Volume
Melezitose	400 mg/mL	3 mL
Treahlose	400 mg/mL	6 mL
Lysine	1.5 mg/mL	3 mL
Glycogen	200 mg/mL	3 mL
Nuclease-free water	NA	q.s.p. 20 mL

Table

Reaction Mix Reagent	Regular mastermix	Gelification mastermix
Oligomix (25X)	1 µL	1 µL
PCR buffer (2X)	12.5 µL	12.5 µL
Gelification Mixture*	-	5 µL
Nuclease-free water	6.5 µL	-
DNA sample*	5 µL	5 µL

*maximum of 20% of the final reaction volume

Table of Materials

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Curitiba (Brazil), December 10st, 2021.

Dear Editors at the Journal of Visualized Experiments,

Please, find enclosed the revised version of the manuscript by Costa and colleagues, entitled **"Producing ready-to-use qPCR for detection of DNA from *Trypanosoma cruzi* or other pathogenic organisms"** to the special edition Current Methods in Trypanosomatids Research.

We are grateful for the comments and suggestions by the editorial team and the reviewers. All were addressed, greatly improving the quality of the manuscript, and a reply to each point is given on this letter.

On behalf of all authors, I wish to thank you in advance for your care and attention in handling this manuscript.

Yours sincerely,

Dr. Alexandre Dias Tavares Costa

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Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Reply. The manuscript was revised and, to the best of our knowledge, is free of grammar and spelling issues. All abbreviations were defined at first use.

2. Please reword the following lines to avoid previously published work: 81-88, 303-307, 312-315.

Reply. The text in those lines has been reworded.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Reply. All text was revised to avoid the use of personal pronouns.

4. Please format in-text journal references to appear as numbered superscripts after the appropriate statement(s) BEFORE punctuation.

Reply. In-text references have been formatted as numbered superscripts.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be

written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a "Note."

Reply. All text was revised to ensure the use of imperative tense. All other protocol information was shown as "Note".

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Reply. The text was revised, and we believe it contains all necessary information for proper replication of the protocol.

Please add more details to your protocol steps:

7. Step 1.1.3/1.2.3/1.3.3/1.4.3: Is it possible to specify the vortex speed?

Reply. Speed of vortex was specified as the maximum allowed by the instrument. The text now reads "Vortex at maximum speed until the powder is solubilized".

8. Step 2.1.2: Please provide details regarding the DNA sample to be used. Also please include the source of the sample in the Table of materials if the sample is obtained commercially.

Reply. In Step 2.1.2, DNA samples are used only for calculations. Details on the DNA sample are now provided in a Note after step 4.3, when DNA samples are added to the reaction tubes.

9. Step 3.4: Please provide a brief NOTE regarding the need of the bentonite clay bag. Also please include the details in the Table of materials.

Reply. The use of bentonite clay bags has been explained in a Note and Table of Materials has been updated.

10. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply. All essential steps were highlighted.

11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manuscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight.

Reply. All highlighted steps were checked to form a cohesive narrative.

12. Figure 2: Please include the description of the X and Y-axes.

Reply. Figure 2 axes were labelled, and a description was added to the figure's legend.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate the Journal names (your format is correct in this aspect).

Reply. References have been formatted per Journal's style.

14. Please ensure that the Table of Materials contains all the details of all the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Reply. The Table of Materials was checked to contain all details of all essential supplies, reagents, or instruments.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The article: "Producing ready-to-use qPCR for detection of DNA from Trypanosoma cruzi or other pathogenic organisms" is a great contribution for those of us who work with real-time PCR.

This procedure may be the way in which molecular techniques could be introduced into the routines of low-income countries, and in areas with greater difficulty to have -20 ºC containers.

Minor Concerns:

1. In the abstract, maybe it is better to indicate Genetically Modified Organisms instead of GMOs.

Reply. The text was changed accordingly.

2. Line 65 - 66: "blood smear microscopy, serology, and real-time PCR (qPCR)…", redaction could be "blood smear microscopy, serology, and molecular tests such as real-time PCR (qPCR)…". Currently, LAMP is also being introduced in the diagnosis of acute infection by T. cruzi.

Reply. We appreciate the point made by the reviewer. The text has been modified to include a mention of isothermal amplification technique as a possible means for diagnosis of Chagas disease.

3. Line 68 - 71: "Because of its sensitivity and specificity, qPCR has been suggested to be used as a monitoring tool for chronic patients or for acute patients undergoing treatment measuring the parasite load in the blood…)." Authors should add: "and surrogate marker of therapeutic failure (Parrado et al, 2019)" Parrado R, Ramirez JC, de la Barra A, Alonso-Vega C, Juiz N, Ortiz L, Illanes D, Torrico F, Gascon J, Alves F, Flevaud L, Garcia L, Schijman AG, Ribeiro I. Usefulness of Serial Blood Sampling and PCR Replicates for Treatment Monitoring of Patients with Chronic Chagas Disease. Antimicrob Agents Chemother. 2019 Jan 29;63(2):e01191-18. DOI:

10.1128/AAC.01191-18.

Reply. We appreciate the point made by the reviewer. Reference was added as requested.

Reviewer #2:

Manuscript abstract:

The protocol by Alexandre Dias Tavares et al; entitled "Producing ready-to-use qPCR for detection of DNA from Trypanosoma cruzi or other pathogenic organisms" describes a gelation technique to effectively preserve qPCR reagents for the detection of T. cruzi satDNA. In my opinion, it is a very interesting method to be able to keep qPCR reagents at room temperature for a few weeks without the need to maintain the cold chain. The introduction is well written and the protocol is well developed and laid out in detail, indicating its advantages as well as its possible limitations.

Major concerns:

In relation to the Representative Results section, (lines 204-210). I am left with doubt about the preparation of the glycogen solution. The authors say that they wait a couple of hours or up to 8-12 hours to settle the bubbles that form in the glycogen solution. Is only two hours enough or is 8-12 hours really necessary? After this time I am left wondering whether more bubbles will form when the glycogen solution is added to the mixture and has to be shaken again. How do you proceed in this respect? The authors should clarify this point.

Reply. We are grateful that the reviewer raised this question, as this is a crucial step of the protocol

and must be crystal clear for the reader. We meant “couple of hours” in a general term, not meaning “two hours”. The text was reworded to clarify this point, and now reads “Therefore, it is very important to let the glycogen solution resting in the refrigerator most of the solution trapped within the bubbles have moved down to the main solution body. Considering the production protocols and the lab routine, gelified plates are kept in the refrigerator overnight (or around 8-12 hours), which results in settling of the solution, making it easy to determine the correct volume and adjust to the desired final volume (Figure 1C).”

When the glycogen solution is used to produce the gelification mixture, it gets diluted, which should decrease the likelihood of bubble generation when vortexing the gelification mixture. However, as pointed out in step 1.5.2, the reagents in the gelification mixture are mixed by end-to-end inversions, which are gentler than vortexing and avoid bubble generation.

Minor concerns:

Can this gelation method be used with other types of enzymes, such as those used in isothermal amplification of DNA or RNA? If the authors have tried it, they could indicate this as well.

Reply. That is an interesting and important question, and we are glad the reviewer asked. Yes, preliminary data from our group using a RT-LAMP reaction for SARS-CoV-2 detection suggest that the gelification method might be used to preserve isothermal amplification reactions. This information is now included in the manuscript in lines 293-296.

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Author(s): Alexandre D.T. Costa, Steffanie S. Amadei, Amanda Bertão-Santos and Ivany Rodrigues

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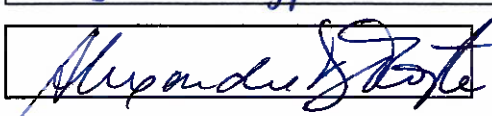
Signature:	 Date: Sep 15th 2021

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