

A protocol for the detection of genetic markers in saliva by polymerase chain reaction without a nucleic acid purification step: examples of SARS-CoV-2 and GAPDH markers.

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Research

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Abstract

Background: The objective of this study was to develop a protocol for direct use of saliva in tests for genetic markers, without purification of nucleic acids. Currently, diagnostic tests use purified nucleic acids from clinical samples. This purification step adds time, cost, and affects the quality of testing. Multiple attempts to remove the purification step were reported.

Results: We report a protocol for the direct detection of genetic markers in saliva. The protocol is based on collection of saliva in a solution containing a detergent and ethanol, and is compatible with isothermal amplification (LAMP), real-time RT-PCR and RT-PCR. SARS-CoV-2 and GAPDH markers were used as reference markers. We observed that mild detergents allow efficient detection of markers (e.g. GAPDH and SARS-CoV-2), while strong detergent, e.g. sodium dodecyl sulfate, inhibited the PCR reaction. Under these conditions, saliva samples can be stored for 24 h at +4⁰C or -18⁰C with preservation of the markers. Storage at room temperature led to deterioration of marker detection. Snap heating of saliva samples at the time of collection, followed by a storage at the room temperature, provided partial protection.

Conclusions: The protocol presented in this report describes collection and storage of saliva for direct detection of genetic markers and is compatible with PCR and LAMP tests.

Introduction

Polymerase chain reaction is a frequently used highly sensitive diagnostic method for detection of nucleic acids. Nucleic acids (NAs) are embedded in various cellular structures, e.g. nuclei, vacuoles or protein-nucleic acids complexes [1]. Accessibility of NAs for amplification is crucial for the performance and sensitivity of a PCR test. Clinical samples contain many different components that may affect PCR reaction, e.g. nucleases and inhibitors [2]. The considerations of DNA accessibility and the complex nature of clinical samples prompted the introduction of a nucleic acid purification step in PCR diagnostic tests. However, this purification step increases the time and cost of each assay, and requires a dedicated laboratory instrumentation [3, 4]. Failure in the purification procedure may also jeopardize an assay [5].

There have been a number of efforts to develop protocols that would not require purification of nucleic acids. Direct detection of genetic markers without any additives to a clinical sample, addition of organic solvents, buffers, detergents, and absorbing materials have all been explored to omit or simplify nucleic acid purification [6–8]. The rationale of these techniques is the releasing and collection of targeted genetic material in a form that can be amplified in a PCR reaction. The success of the reported methods vary. For example, a direct detection of SARS-CoV-2 in nasopharyngeal swabs sample media has been reported, e.g. in Virocult, Transwab [8]. This direct detection however required lysis and heat-inactivation of swab samples. Addition of detergents to the sample collection media has also been reported. Detergent-mediated lysis releases the NAs from various complexes and structures. However, detergents may have a detrimental effect on the stability of the reverse transcriptase and/or DNA polymerase used in PCR tests [6, 8]. An approach of snap-heating swab samples immediately upon collection has been

reported [8]. The rationale for this technique is the denaturation of proteins, including nucleases, upon heating the sample to between 60⁰C and 120⁰C for few seconds. The drawback of this approach is that the heating of swab samples may induce RNA degradation.

Clinical samples used for PCR diagnostic analysis differ in their composition, based on the origin of the sample. Blood, plasma, serum, buccal swabs, nasopharyngeal swabs and saliva are the most frequently used clinical materials. Among these, saliva is most suitable for self-collection. Collection of blood or swabs require trained personnel. Therefore, saliva has been extensively explored as a source of clinical samples. For the ongoing COVID-19 pandemic, saliva is used as a clinical source for testing [9–15]. The variability of saliva composition was of concern for reliability of tests, e.g. presence and quantity of marker-containing material, chemical and enzymatic impact on intactness of markers, protocols for extraction and stabilization of the markers. However, recent reports show promising results, and confirm that saliva has to be considered as a material for testing [9–15].

The ongoing COVID-19 pandemic raised the issue of efficient detection of the virus. Typically, thousands of tests are performed in a single testing laboratory. The burden of a large number of tests accentuated the efforts to simplify the test protocol. Here we report that genetic markers can be detected in saliva by collection of a sample in a solution containing the mild detergents Triton X-100 and Tween 20. Tests with markers for SARS-CoV-2 and human GAPDH confirmed the efficacy of using detergent-containing solutions for collection of saliva samples. The protocol described in this report significantly simplifies PCR- and LAMP-based tests on clinically relevant biological samples.

Results

Direct PCR on saliva collected with a sample solution containing detergents and ethanol.

To test different extraction components, we tested the following sampling solutions: a) 1.0% SDS, b) 1.0% Triton X-100, c) 1.0% Tween 20, d) 40% ethanol and e) water. Saliva was collected in these solutions at a 1:1 ratio. DNA templates for SARS-CoV-2 (TS22) or GAPDH (TP1) were added to the saliva samples and/or tested as annotated in Figure 1.

The GAPDH TP1 template was added to the sampling solutions in 2 concentrations, 1.5x10⁻¹⁰ M and 1.5x10⁻¹¹ M, respectively. The samples were handled at room temperature. One microL of the sample:solution mixture was used for each PCR reaction. On average, the time for preparation of the reactions was 20-35 min. The PCR cycling protocol is described in Table 2. PCR products were separated by agarose electrophoresis and the gels were stained with SYBR Safe (Figure 1A). Specific PCR products were quantified using ImageJ (Figure 1). Quantification showed that SDS strongly inhibited the PCR reaction, while Triton X-100, Tween 20 and ethanol did not affect the PCR. Similar results were obtained when testing the SARS-CoV-2 marker; the SARS-CoV-2 template (TS22) and specific primers were used (Figure 1B). SDS inhibited the PCR reaction, while the SARS-CoV-2 marker was detected in the samples

collected in Triton X-100 and Tween 20 in 40% ethanol. Therefore, we proceeded with the sampling solution containing 1.0% Tween 20 with 40% ethanol.

This solution was found to be compatible with real-time PCR protocols and with the LAMP assay (Figure 1C,D). For the LAMP assay, the template was a SARS-CoV-2 sequence, with 4 primers targeting 6 sites in the sequence. DNA amplification was also monitored by the change of color of the reaction mixture from violet to yellow; cresol red was used as a pH sensing dye. Analysis of generated DNA products by gel electrophoresis showed similar quantities of DNA generated from the template mixed with the saliva in the sample solution and the template in water (Figure 1C).

Real-time PCR is frequently used in diagnostic. We observed that saliva collected in the sample solution did not interfere with real-time PCR tests (Figure 1D). We used two protocols of qRT-PCR. The first was a Taqman protocol detecting generation of the fluorescent dye FAM (with a quencher IowaBlack) and the second was based on detection of generated PCR products with SYBR Green. Ct for the samples with and without saliva was similar, i.e. 26 (Figure 1D). Titration of the template detection showed that both assays could detect as little as 10 molecules in 1 ml of the sample used in the 25 ml reaction, This gives the limit of detection by the qRT-PCR assay corresponding to 1×10^4 molecules per ml.

Thus, RT-PCR, two qRT-PCR detection methods (SYBR Green and FAM/IowaBlack) and LAMP assays showed that saliva can be used for the direct detection, and that 1% Tween 20 and 40% ethanol solution is suitable for collection of saliva for testing.

Storage conditions: +4⁰C or freezing are recommended

To evaluate the impact of storage on the detection, saliva samples with added DNA templates were stored for different periods of time at different temperatures. Samples were stored for 24 h, 5 h, or 0.5 h before use for PCR amplification (Figure 2A, B). Testing is recommended within 24 h of a sample collection, and therefore storage for more than 24 h was not tested. We observed a decrease of the signal following 5 h storage at room temperature (20⁰-22⁰C). After 24 h storage at room temperature, the signal decreased by more than 90 %. Storage of the samples at +4⁰C or -18⁰C did not affect detection of markers. These temperature conditions are recommended for clinical use. Freezing samples may be complicated at the sites of collection. Therefore, storage at +4⁰C is a good alternative the storage and transportation of the samples.

At room temperature (20⁰-22⁰C) many enzymes, including nucleases, are fully active. Therefore, the decrease in efficacy of detection after storage at room temperature for 5 and 24 h was expected. Our data suggest that the storage at room temperature for longer than 1 h should be avoided.

Snap-heating of clinical samples has been used to preserve degradation [6,8]. Short bursts of heating to 100-120⁰C denatures proteins and protects sample from degradation, as the degrading enzymes are proteins. We observed that 5 minutes heating to 80⁰C followed by storage at room temperature, prevented sample degradation to a significant extend (Figure 2A, B). Thus, snap-heating can be used if there is no

possibility to store samples at +4⁰C or below. Therefore, the recommended storage and transportation conditions are +4⁰C or below.

Control experiments with the saliva samples included sterility tests and the separation of saliva samples on SDS-polyacrylamide gels to monitor the protein pattern in samples (Supplementary Figure S1). These experiments show that the recommended sample solution (1% Tween 20, 40% ethanol in water) prevented microbial growth. The electrophoresis profile of saliva was similar to reported saliva profiles [16]. See supplementary figure S1 for examples of these control experiments.

Detection of endogenous cellular target

Detection of genetic markers requires that they are accessible to primers. Most genetic markers are found in complexes with other molecules, e.g. proteins. Cellular DNA and RNA form complexes with proteins, and viral DNA/RNA is contained within capsids [1]. For detection by PCR, nucleic acids have to be released from these complexes. To explore how incubation with the sample solution may affect access to endogenous targets, we decided to test if we could detect endogenous human GAPDH (Figure 2C). Cultured human breast adenocarcinoma (MCF7) and renal carcinoma (ACHN) cells were harvested in suspension. Both types of cells express GAPDH, a housekeeping gene constitutively expressed in cells and tissues (<https://www.ncbi.nlm.nih.gov/gene/2597>). Intactness of the cells was monitored under the microscope. The cell suspension was mixed with saliva and the sample solution, or the cell suspension was mixed with water, as annotated in Figure 2C). The ratio was 1:1:2 for cells:saliva:sampling solution, respectively. Under these conditions, we were able to detect endogenous GAPDH in cell extracts with or without saliva in sample solution, with the same sensitivity that obtained with synthetic DNA template of GAPDH (TP1). Figure 2C shows an example with MCF7 cells; similar results were obtained with ACHN cells. For the PCR reaction, reverse transcriptase was used to generate cDNA from cellular GAPDH mRNA. Two concentrations of the cell extract with and without saliva in the sample solution were tested, i.e. 1x and 100x diluted cell extract, annotated as 1.0 and 0.01 respectively (Figure 2C). Detection of endogenous GAPDH in the presence of saliva shows that the sample solution can be used for detection of intracellular genetic markers.

Discussion

Omitting nucleic acid purification may significantly facilitate PCR-based testing. However, the complexity of clinical samples, and the presence of nucleases, as well as the complexing of nucleic acids with proteins complicate efficient direct detection. The protocol described here overcomes the problems associated with nucleic acid purification, preservation and accessibility of genetic markers for testing. Direct detection of genetic markers removes a costly and time-consuming purification step from the testing protocol [6,8,9]. The composition of the sample solution described here promotes the preservation of genetic markers and also allows for storage and transportation of clinical samples. This is of great importance, since many testing sites do not have access to advanced instrumentation. Saliva is also

easier to collect as compared to other types of samples. Saliva can be self-collected, and has been extensively explored as a source of testing [9].

The collection solution described in this report contains Tween 20 and ethanol. Mixing saliva with the sampling solution at a 1:1 ratio results in a solution containing 0.5% of detergent and 20% ethanol. The concentration of Tween 20 was sufficient to relax protein complexes without affecting the enzymes in the PCR reaction (Figure 1). SDS, in contrary to Tween 20, is a more potent denaturing detergent (<https://pubchem.ncbi.nlm.nih.gov/compound/3423265>), which was reflected in the inhibition of the PCR reaction when the sample solution contained SDS (Figure 1). Tween 20 is used in extraction of proteins, and is known as a mild denaturing agent (<https://pubchem.ncbi.nlm.nih.gov/compound/Polysorbate-20>). This feature of Tween 20 is of benefit for the extraction and stabilization of genetic markers (Figures 1 and 2).

Saliva contains microorganisms that are present in the oral cavity. The addition of ethanol blocked microbial growth (Supplementary Figure S1). Therefore, the presence of a mild denaturant and ethanol protects from microbial growth and facilitates accessibility of the targeted markers.

The storage of samples for the time between collection and analysis is of importance for successful testing. Saliva contains enzymes and chemical entities that may affect stability of the markers. Approaches to preserve sample integrity include freezing, keeping at cold, and chemical or thermal stabilization [6,8,9]. Storage at room temperature, i.e. 20⁰C and higher, is not recommended. We observed that the storage of samples at +4⁰C or -18⁰C for 24 h preserved markers (Figure 2). Storage at room temperature resulted in the degradation of the markers already after 5 h. Snap-heating at +80⁰C for 5 min immediately following sample collection is aimed at denature enzymes in saliva [6,8]. Stabilization of the genetic marker after a snap-heating was observed (Figure 2A,B), although the efficiency of detection was lower as compared to storage at +4⁰C or -18⁰C.

We observed that the direct use of saliva without nucleic acid purification was compatible with standard protocols of real-time PCR and LAMP amplification (Figure 1C, D). This compatibility extends the use of our protocol to different SARS-CoV-2 detection methods.

Conclusion

This report describes a protocol for successful use of saliva for direct detection of genetic markers, and omitting the nucleic acids purification step. The protocol reports optimization conditions for saliva collection, storage and testing.

The protocol describes collection of saliva in a solution containing Tween 20 and ethanol, storage conditions (+4⁰C or frozen), and shows compatibility with PCR and LAMP methods. Our report describes also crucial practical moments in saliva collection and storage that can affect results, e.g. troubleshooting by comparison with other solutions and detergents, deviations from the optimal storage

conditions and how that may affect results. Such troubleshooting would be of help for implementation of the reported here protocol. Robustness and simplicity of the reported here protocol is of advantage for its clinical use.

Materials And Methods

Primers and templates

SARS-CoV-2 sequence (NC_045512) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; NM_001256799.3) were used to design primer-template pairs. The sequences of templates and primers are presented in Table 1. Primers and targets were ordered from Integrated DNA Technologies (www.idtdna.com), Twist Bioscience (www.twistbioscience.com), and SynBio Technologies (<https://www.synbio-tech.com/>). Reactions were performed with Platinum II Hot-Start PCR Master Mix (Invitrogen by Thermo Fisher Scientific; 14000-013) and AccessQuick RT-PCR System (Promega; A1702) kits. Chemicals were obtained from Sigma Aldrich and ThermoScientific/Merck. All chemicals were of analytical grade.

Ethical considerations and saliva collection

Saliva samples were collected from laboratory volunteers after obtaining a written informed consent. The ethical permit was obtained from the Qatar University Institutional Review Board; the experimental work was performed under QU permit number QU-IBC-2019/023. No personal data were collected, and only a random number was assigned to the sample. Saliva was self-collected, by spitting approximately 1 ml of saliva in a 50-ml sterile laboratory tube containing 1 ml of the sample solution. Collected saliva was spiked with templates of the markers as described in corresponding sections below.

PCR reactions

Programs of PCR reactions are described in Table 2. The temperatures and number of cycles were optimized for the primers and templates in the absence of saliva. SureCycler 8800 (Agilent) and PTC-100 (MJ Research) cyclers were used for PCR amplification. The PCR reaction was set with the Platinum Hot Start PCR 2X Master Mix (Invitrogen/ThermoFisher Scientific; 14000-013). Reverse transcriptase PCR reaction was performed with AccessQuick RT-PCR kit purchased from Promega (cat. no. A1702). PCR products were separated by agarose electrophoresis (ThermoFisher Scientific), using 2% agarose (UltraPure Agarose, Invitrogen) in 1x TBE. The gel was stained with SYBR Safe DNA stain (Invitrogen), and the separated PCR products were visualized using the iBright CL1000 imaging system (ThermoFisher Scientific). DNA size markers were Trackit 1kb Plus DNA ladder (Invitrogen). Quantifications were performed using ImageJ [17].

Real-time PCR

Real time PCR was performed with QuantStudio 6 Flex Real-Time PCR System instrument (Applied Biosystems, ThermoFisher Scientific). We performed real-time PCR tests with the SYBR Green and with a

fluorescent dye. Reactions were set up as for the regular PCR reactions described above. For detection with SYBR Green, the stain was added to the reaction mixture (final concentration 1 mM). The TaqMan Reagents protocol was set to 40 cycles at 92⁰C for 5 sec and 55⁰C for 30 sec. The Ct of amplifications were calculated using the QuantStudio 6 Flex System. Melting curves were also collected. For the real-time PCR with the fluorescence dye, a middle primer with a FAM reporting dye and IowaBlack quencher was added to the reaction mixtures. The SYBR Green Reagent protocol was set to 40 cycles at 92⁰C for 5 sec and 55⁰C for 30 sec. Data were analyzed in QuantStudio 6 Flex System. Following the real-time PCR analysis, all reactions were subjected to the gel electrophoresis to monitor generated products, as described above in the “PCR reactions” section.

Loop-mediated isothermal amplification (LAMP)

LAMP test was used for detection of 2 different regions of SARS-CoV-2. LAMP amplification was performed with Bst 3.0 polymerase (New England Biolabs) for 30-60 min at 65⁰C. Amplification was performed with 4 primers targeting 6 regions in the SARS-CoV-2 genome, and with a synthesized target representing SARS-CoV-2 region. Primers and targets were obtained from Integrated DNA Technologies (www.idtdna.com), Twist Bioscience (www.twistbioscience.com), and SynBio Technologies (<https://www.synbio-tech.com/>). Detection was with 50 mM cresol red in the reaction mixture, by monitoring change of color from violet to yellow. Generation of DNA products was monitored by the agarose gel electrophoresis as described above.

Declarations

Ethical approval: This work was performed under an IBC permit from Qatar University (QU-IBC-2019/023). Self-collected saliva was provided by healthy volunteers upon signing an informed consent form of the Qatar University Institutional Review Board.

Consent for publication: Not applicable. All authors approved the manuscript.

Availability of data and materials: All data are available upon request. All data generated and analyzed during the study are included in this published article.

Competing interests: All authors declare that they have no competing interests.

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Authors contributions: S.K. performed experiments, participated in writing and approval of the manuscript. J.E. assisted with experiments, participated in writing and approved the manuscript. N.S. participated in design of assays, assisted with technological input, participated in writing and approval of the manuscript. S.S. designed and supervised the project, performed experiments, wrote and approved the manuscript.

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Abbreviations

LAMP, isothermal loop amplification; NAs, nucleic acids; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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Tables

Tables 1-2 are available as a download in the Supplementary Files section.

Figures

Figure 1

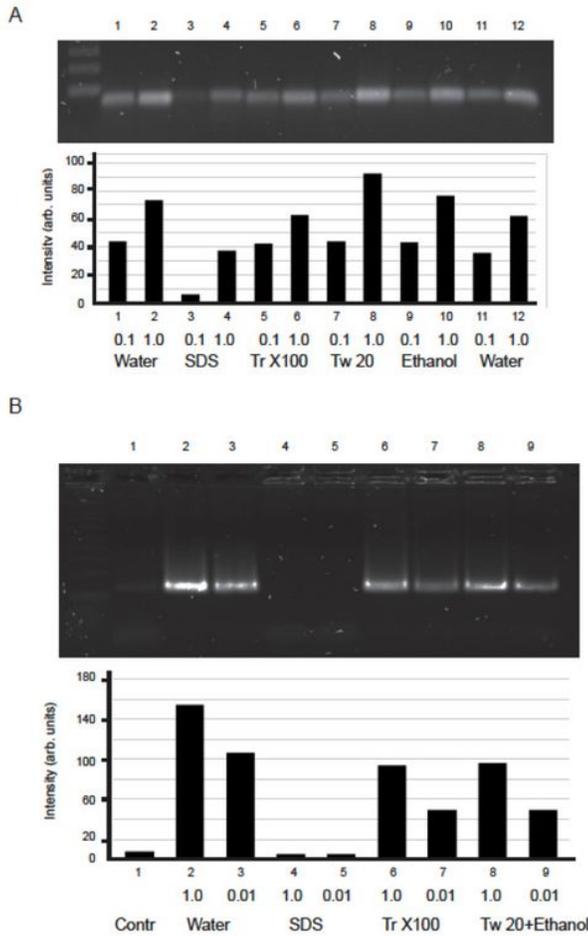


Figure 1 C

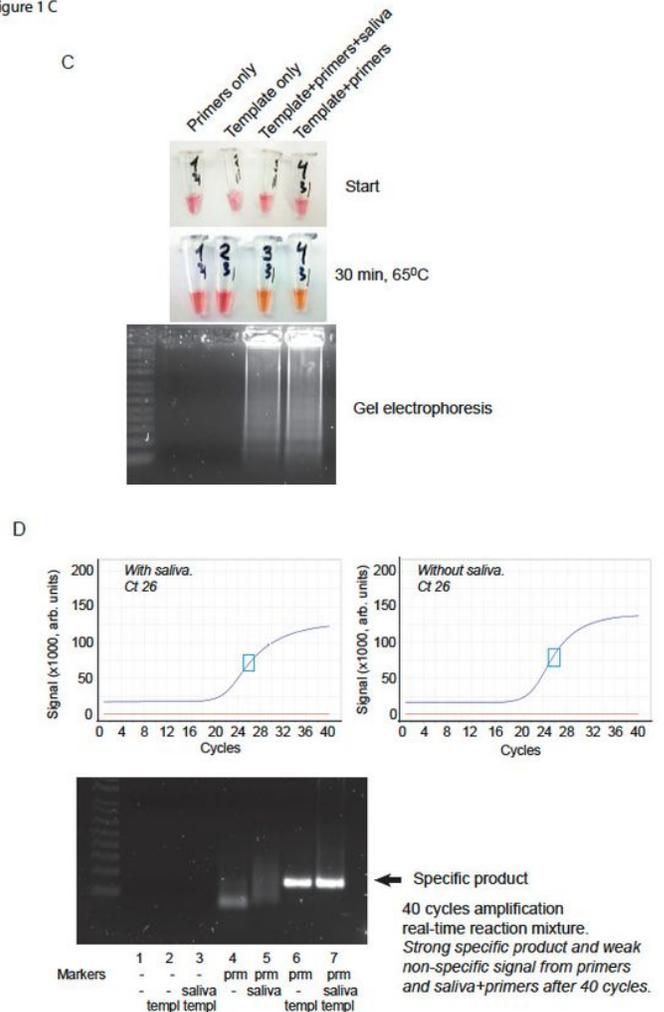


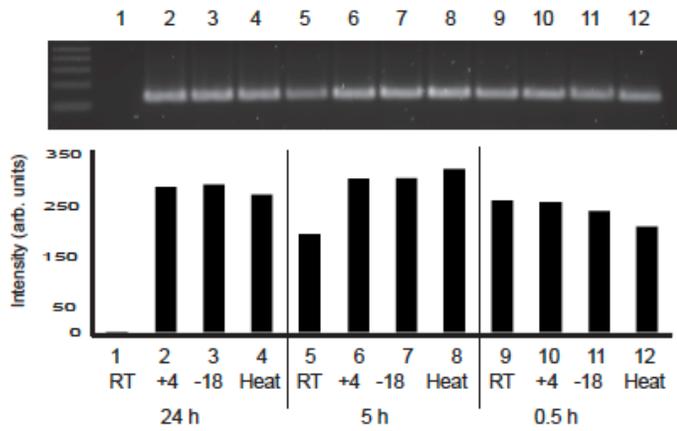
Figure 1

Direct detection of genetic markers in saliva collected with detergents and ethanol. SDS inhibited and Tween 20 and Triton X-100 allowed detection of GAPDH (A) and SARS-CoV-2 (B) markers by a PCR test. Experimental conditions were as annotated. 1.0 and 0.1 refer to concentration of the template; 1.0 corresponds to 1×10^5 molecules/ml, 0.1 corresponds to 1×10^4 molecules/ml. The template was diluted in water (Water), sodium dodecyl sulfate (SDS) Tritox X100 (Tr X100), Tween 20 (Tw20) or ethanol (Ethanol). Images show visualization of the PCR product in an agarose gel. The lower part of the figure show quantification of the PCR product. C) Isothermal amplification (LAMP) was performed with saliva samples for detection of the SARS-CoV-2 markers. The upper panel shows the change in color of the reaction mixtures for samples containing SARS-CoV-2 markers. The lower panel shows separation of the amplification products in an agarose gel. D) Saliva did not interfere with detection of SARS-CoV-2 markers by a real-time PCR. Two amplification curves for samples with or without saliva are shown for conditions #6 and #7 shown in the electrophoresis image. Ct values for both are 26. The gel image shows products of the real-time PCR reactions after 40 cycles stained with the SYBR Green dye. The conditions are annotated with numbers 1 to 7. Annotations are for addition of primers "prm", template

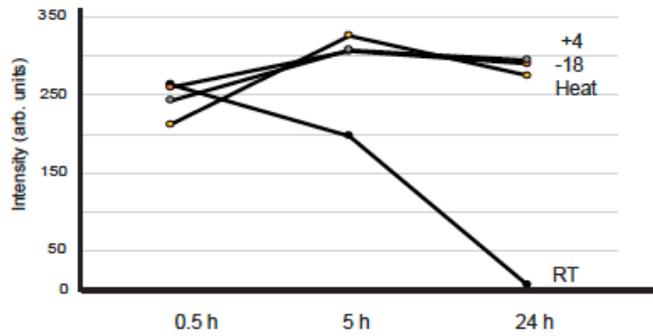
“temp”, and saliva in sample solution “saliva”. The specific product is indicated by the arrow. Representative experiments out of total of 3 (A), 6 (B), 5 (C) and 4 (D) are shown.

Figure 2, A, B, C

A



B



C

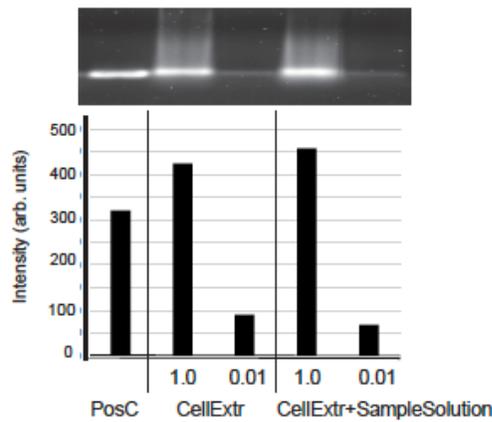


Figure 2

Impact of different storage conditions and the detection of endogenous markers. SARS-CoV-2 marker was incubated with saliva for 30 min, 5 h and 24 h at room temperature (200C-220C), +40C and -180C, as indicated in panels A and B. A) The upper panel shows the visualized PCR product, and the lower panel

represents corresponding quantification with ImageJ in arbitrary units. Sample numbers in the gel electrophoresis panel correspond to the numbers annotating quantification. Annotations are as follow: RT - room temperature; +4 - +40C; -1 -, -180C; Heat - snap-heating. B) Graphical presentation of detection of the SARS-CoV-2 marker after storage up to 24 h. A significant decrease of the marker detection was observed after 5 h at room temperature, with further reduction at 24 h. Annotations are as in the panel A. C) The sample solution allows detection of endogenous GAPDH. Suspension of MCF7 human cells, annotated as CellExtr 1.0 for 1×10^6 cells/ml and 0.01 for 1×10^4 cells/ml for 100x diluted sample, was mixed with the sample solution and saliva, as annotated. Endogenous GAPDH was detected by reverse transcriptase PCR. Synthetic GAPDH DNA was used as a positive control (PosC), with the GAPDH template (TP1) added. Annotations 1.0 and 0.01 refer to concentration of the cell extract; 1.0 corresponds to 1×10^6 cells/ml, 0.01 corresponds to 1×10^4 cells/ml. Quantification of the generated PCR product with ImageJ is shown in the lower panel. Representative experiments out of total 3 (A, B) and 4 (C) are shown.

Supplementary Files

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