Протокол детекції генетичних маркерів у слині, використовуючи полімеразну ланцюгову реакцію без очистки нуклеїнових кислот: тестування SARS-CoV-2 та GAPDH маркерів

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Вступ. Діагностичні тести на основі полімеразної ланцюгової реакції (ПЛР) використовують очищені нуклеїнові кислоти з клінічних зразків. Етап очищення нуклеїнових кислот потребує часу, додає вартість та впливає на якість тестувань. Метою цього дослідження є розробка протоколу використання слини в тестах на генетичні маркери без очищення нуклеїнових кислот.

Методи дослідження. Для виявлення генетичних маркерів без очищення нуклеїнових кислот використовували ревертазну ПЛР (RT-PCR), ПЛР в реальному часі (qRT-PCR) та тести ізотермічної ампліфікації (LAMP).

Результати. Ми розробили та оптимізували протокол для виявлення генетичних маркерів у слині. Протокол заснований на зборі слини в розчині, що містить детергенти та етанол, і є сумісний з ізотермічною ампліфікацією, ревертазною ПЛР, та ПЛР в реальному часі. Маркери SARS-CoV-2 та GAPDH використовувались як еталонні маркери. Оптимізація протоколу показала, що м'які солюбілізуючі детергенти (e.g. Тритон X-100/Triton X-100 або Твін-20/Tween-20), дозволяють ефективно виявляти маркери GAPDH та SARS-CoV-2, тоді як сильні детергенти, наприклад, додецилсульфат натрію, пригнічував реакцію ПЛР. Зразки слини, зібрані в розчині Твін-20, та етанолу, можуть зберігатися протягом 24-х годин при +4°C або –180°C із збереженням маркерів інтактними. Зберігання при кімнатній температурі призводить до погіршення стабільності маркерів. Швидке нагрівання зразків слини під час збору з подальшим зберіганням при кімнатній температурі забезпечувало частове збереження стабільності маркерів.

Висновки. Протокол описує збір та зберігання слини для виявлення генетичних маркерів. Цей протокол є сумісний з тестами ПЛР та ізотермічної ампліфікації.

***Ключові слова**:* слина, тестування, RT-PCR, LAMP.

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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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Introduction. Polymerase chain reaction (PCR)-based diagnostic tests use purified nucleic acids (NAs) from clinical samples. The NAs purification step adds time, cost, and affects the quality of testing. 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.

Methods. PCR, real-time RT-PCR and isothermal amplification tests were used for direct detection of genetic markers, without purification of nucleic acids.

Results. We report a protocol for the direct detection of genetic markers in saliva. The protocol is based on a 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 external reference and intracellular endogenous markers, while strong detergent, e.g. sodium dodecyl sulfate, inhibited the PCR reaction. Under these conditions, saliva samples can be stored for 24 h at +4°C or –18°C with the preservation of markers. Storage at room temperature led to the deterioration of marker detection. Snap heating of saliva samples at the time of collection, followed by storage at room temperature, provided partial protection.

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

Keywords: saliva, sample collection, direct detection, RT-PCR, LAMP.

Introduction

Polymerase chain reaction is a frequently used highly sensitive diagnostic method for detecting 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 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 the purification of nucleic acids. Direct detection of genetic markers without any additives to a clinical sample, the 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 release and collection of targeted genetic material in a form that can be amplified in a PCR reaction. The success of reported methods varies. For example, 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. The addition of detergents to the sample collection media has also been reported. Detergent-mediated lysis releases 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 60°C and 120°C 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. The collection of blood or swabs requires trained personnel. Therefore, saliva has been extensively explored as a source of clinical samples. In the ongoing COVID-19 pandemic, saliva is used as a clinical source for testing [9–15]. The meta-analysis by Butler-Laporte and colleagues showed high sensitivity (74–91 %) and specificity (98–99 %) of SARS-CoV-2 detection in saliva [16]. The variability of saliva composition was of concern for the reliability of tests, e.g., presence and quantity of marker-containing material, chemical and enzymatic impact on the 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 [9–16].

Here we report that genetic markers can be detected in saliva by collecting a sample in a solution containing mild detergents Triton X-100 and Tween 20. Tests with markers for SARS-CoV-2 and endogenous intracellular human GAPDH confirmed the efficacy of using detergent-containing solutions for the collection of saliva samples. The protocol described in this report significantly simplifies PCR- and LAMP-based tests by direct detection of genetic markers in saliva.

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.

Table 1

Primers and templates used in the study

Target	Name	Sequence (5′ to 3′)
SARS-CoV-2	Sp2a (forward)	catctgatttggctactaac
	Sp2b (reverse)	cacaagcacaggttgagata
	Template TS22	catctgatttggctactaacactaacatctttggcactgtttatgaaaaactcaaacccgtccttgatt ggcttgaagagaagtttaaggaaggtgtagagtttcttagagacggttgggaaattgttaaatttat ctcaacctgtgcttgtg
GAPDH	P1a (forward)	tgatgcttttcctagattat
	P2b (reverse)	atgagttaaaagcagccctg
	Template TP1	tgatgcttttcctagattatggtcgtattgggcgcctggtcaccagggctgcttttaactcat
Real-time PCR	Sp3a (forward)	catgctatacatgtctctgg
	Sp3b (reverse)	cgcactagaataaactctga
	Sp3FAM (FAM dye, middle)	gaggtttgataaccctgtcc
	Template TS33	catgctatacatgtctctgggaccaatggtactaagaggtttgataaccctgtcctaccatttaatg atggtgtttaaaagtctgtgaatttcaattttgtaatgatccatttttgggtgtttattaccacaaaaaca acaaaagttggatggaaagtgagttcagagtttattctagtgcg
LAMP	MF301	ggctaactaacatctttggc
	MF1201	gtctctaagaaactctacaccttccaaacgattagcaaaactgtttatgaaaaactcaaacc
	MB1201	tatctcaacctgtgcttgtgaaaaaacgattagcaaaagaatgtctgaacactctcct
	MB301	gtcagcacacaaagccaa
	Template	gcagtggctaactaacatctttggcactgtttatgaaaaactcaaacccgtccttgattggct tgaagagaagtttaaggaaggtgtagagtttcttagagacggttgggaaattgttaaatttat ctcaacctgtgcttgtgaaattgtcggtggacaaattgtcacctgtgcaaaggaaattaagga gagtgttcagacattctttaagcttgtaaataaatttttggctttgtgtgctgactctatc

Ethical considerations and saliva collection Saliva samples were collected from laboratory volunteers after obtaining 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 markers as described in corresponding sections below.

Table 2

PCR protocols used in the study

SARS-CoV-2 PCR protocol (real-time version has 40 cycles; see the text)	1. 95^{0}C 120 sec 2. 95^{0}C 10 sec 3. 55^{0}C 40 sec 35 cycles 2->3
GAPDH PCR protocol	1. 95^{0}C 120 sec 2. 95^{0}C 10 sec 3. 55^{0}C 30 sec 25 cycles 2->3
Reverse-transcriptase PCR protocol	1. 40^{0}C 40 min 2. 95^{0}C 60 sec 3. 95^{0}C 10 sec 4. 55^{0}C 40 sec 35 cycles 3->4
LAMP protocol	65^{0}C for 20 to 60 min

PCR reactions

Programs of PCR reactions are described in Table 2. Temperatures and number of cycles were optimized for 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). The 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 tool (Applied Biosystems, ThermoFisher Scientific). We performed real-time PCR tests using the SYBR Green and with a fluorescent dye. Reactions were set as for regular PCR reactions described above. For detection using SYBR Green, the stain was added to the reaction mixture (final concentration 1 μM). The TaqMan Reagents protocol was set to 40 cycles at 92^0C for 5 sec and 55^0C for 30 sec. The Ct of amplification were calculated using 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 reaction mixtures. The SYBR Green Reagent protocol was set to 40 cycles at 92°C for 5 sec and 55°C for 30 sec. Data were analyzed in QuantStudio 6 Flex System. Following the real-time PCR analysis, all reactions were subjected to gel electrophoresis to monitor generated products, as described above in the «PCR reactions» section.

Loop-mediated isothermal amplification (LAMP)

LAMP test was used to detect 2 different regions of SARS-CoV-2. LAMP amplification was performed with Bst 3.0 polymerase (New England Biolabs) for 30–60 min at 65°C. Amplification was performed with 4 primers targeting 6 regions in the SARS-CoV-2 genome, and with a synthesized target representing the 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 μM cresol red in the reaction mixture, by monitoring change of color from violet to yellow. The generation of DNA products was monitored by the agarose gel electrophoresis as described above.

Results

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

To test different extraction components, we used 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 and SARS-Cov-2 TS22 templates were added to sampling solutions in 2 concentrations, $1.5x10^{-10}$ M and $1.5x10^{-11}$ M, respectively. This provides a robust detection with 10 marker molecules per test reaction, Template titration tests showed that the copy detection threshold was at 1.0x10^2 copies per milliliter. Samples were handled at room temperature. One microL of the sample: solution mixture was used for each PCR reaction. On average, the time to prepare reactions was 20–35 min. The PCR cycling protocol is described in Table 2. PCR products were separated by agarose electrophoresis and 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 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 the reaction mixture color 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 detecting 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. The titration of detection limit by the qRT-PCR assay showed the range similar to RT-PCR and LAMP tests, i.e. 1x10^2 molecules per milliliter.

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 collecting saliva for testing. The copy number threshold of detection for tests was determined as 1.0x10^2 copies per milliliter, with a robust detection of 10 marker molecules per a test-reaction.

Storage conditions: +4°C or freezing are recommended

To evaluate the impact of storage on the detection, saliva samples with added DNA templates were stored for different periods at different temperatures. Samples were stored for 24 h, 5 h, or 0.5 h before using PCR amplification (Figure 2A, B). Testing is recommended within 24 h of 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 (20–22°C). After 24 h storage at room temperature, the signal decreased by more than 90 %. Storage of samples at +4°C or –18°C did not affect the detection of markers. These temperature conditions are recommended for clinical use. Freezing samples may be complicated at the sites of collection. Therefore, storage at +4°C is a good alternative for the storage and transportation of samples.

At room temperature (20–22°C), many enzymes, including nucleases, are fully active. Therefore, decreased efficacy of detection after storage at room temperature for 5 and 24 h was expected. Our data suggest that 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 100-120^{0}C denatures proteins and protects the sample from degradation, as degrading enzymes are proteins. We observed that 5 minutes of heating to 80°C followed by storage at room temperature, prevented sample degradation to a significant extent (Figure 2A, B). Thus, snap-heating can be used if there is no possibility to store samples at +4°C or below. Therefore, the recommended storage and transportation conditions are +4°C or below.

Control experiments with saliva samples included sterility tests and 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 [18]. See supplementary figure S1 for examples of these control experiments.

Detection of endogenous intracellular 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). The intactness of cells was monitored under the microscope. The cell suspension was

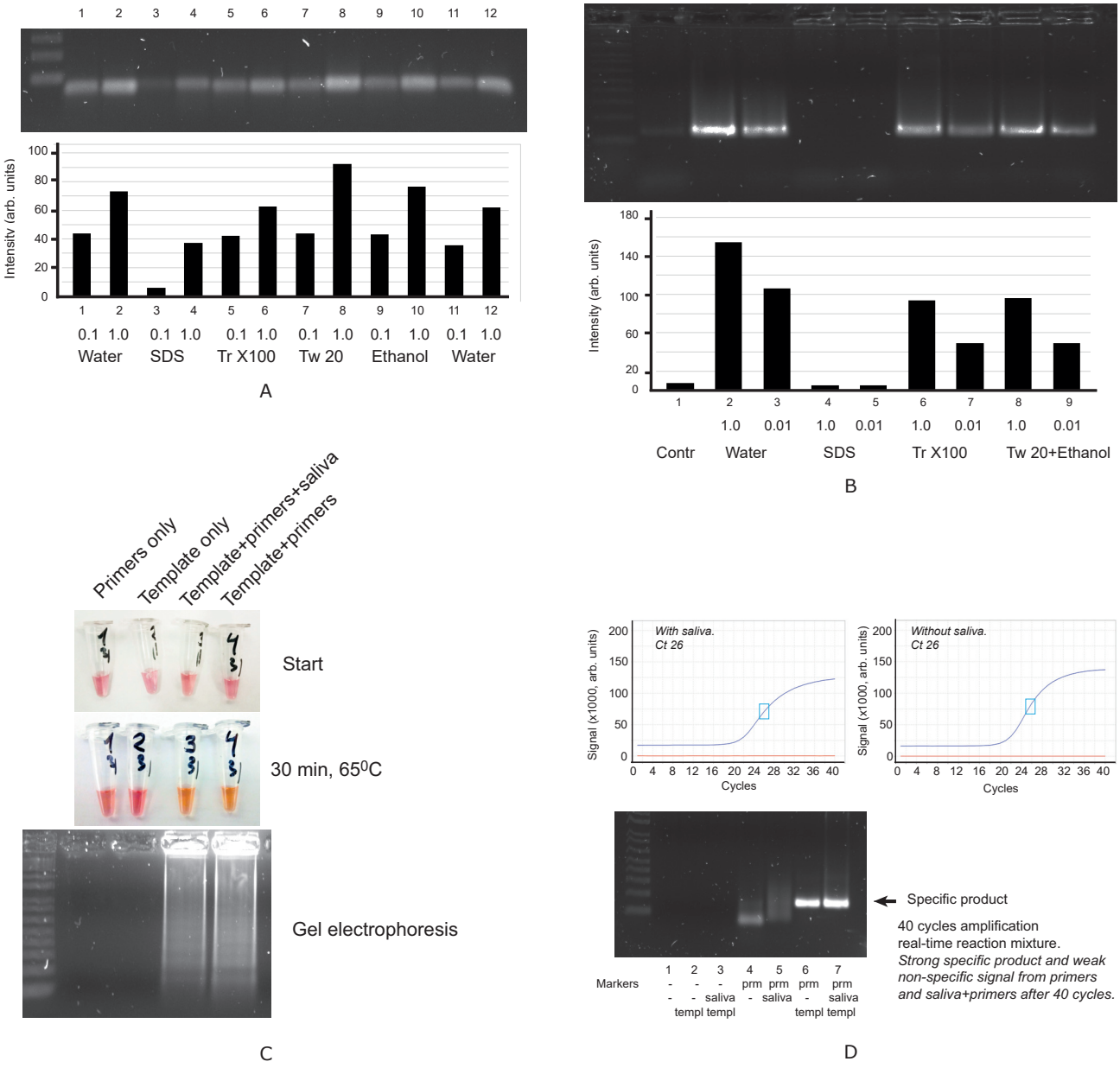


Figure 1. Direct detection of genetic markers in saliva collected with detergents and ethanol

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 1x10^5 molecules/ml, 0.1 corresponds to 1x10^4 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 a 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 detecting 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 the separation of amplification products in an agarose gel. D) Saliva did not interfere with the 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 displayed 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 the addition of primers «prm», template «temp», and saliva in sample solution «saliva». The specific product is indicated by the arrow. Representative experiments out of a total of 3 (A), 6 (B), 5 (C) and 4 (D) are shown.

mixed with saliva and 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 the sample solution, with the same sensitivity that was obtained with the 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 to detect intracellular genetic markers.

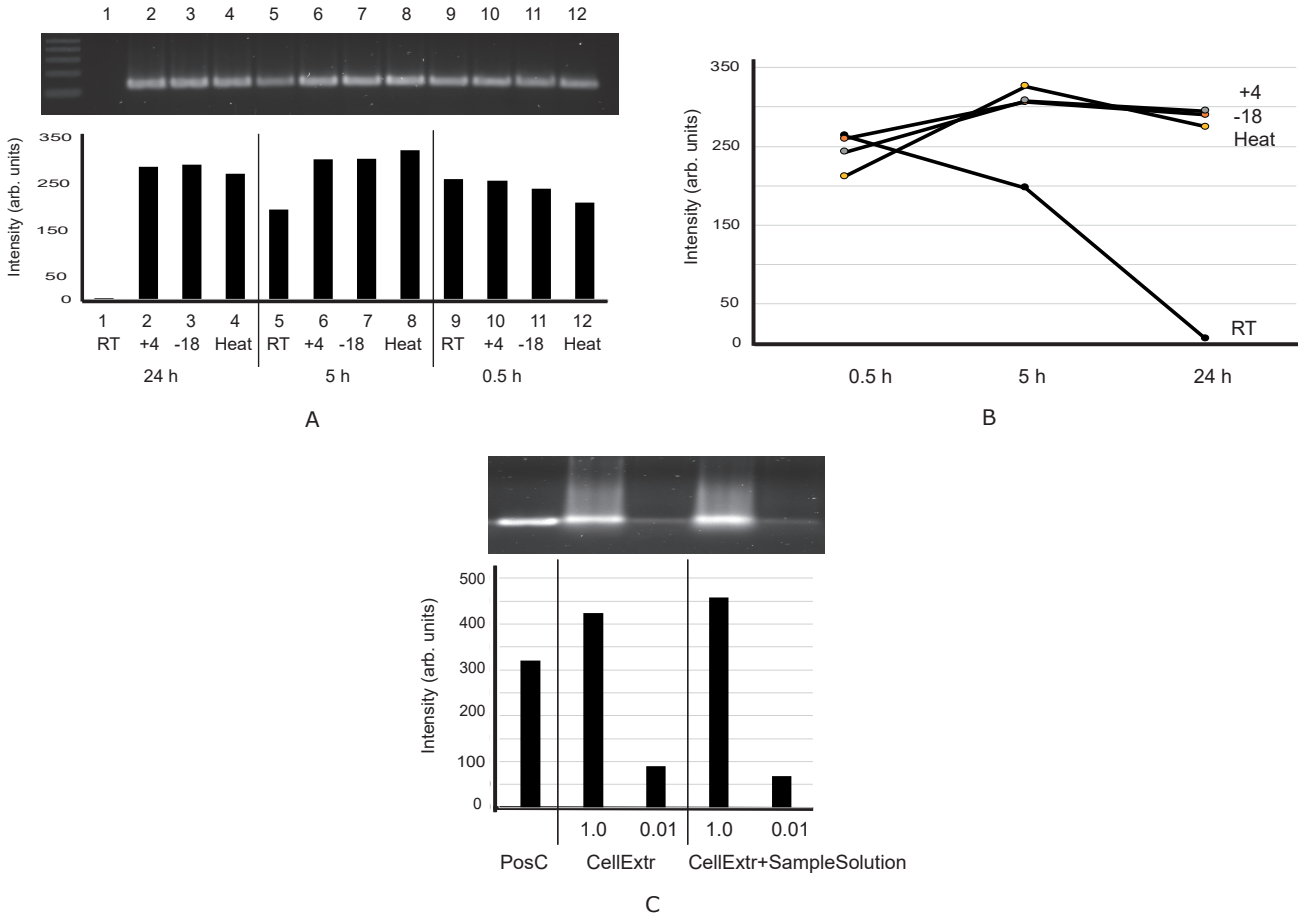
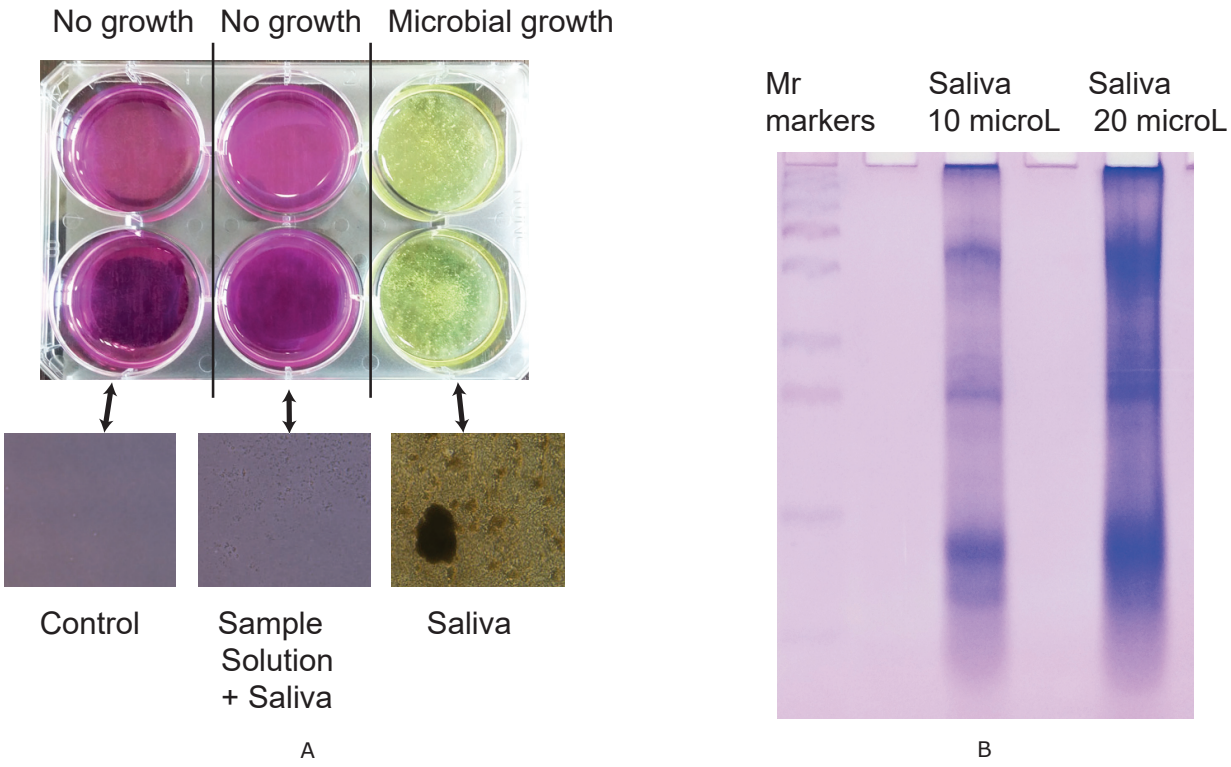


Figure 2. Impact of different storage conditions and the detection of endogenous markers.

SARS-CoV-2 marker was incubated with saliva for 30 min, 5 h and 24 h at room temperature (20–22°C), +4°C and –18°C, as indicated in panels A and B. A) The upper panel shows the visualized PCR product, and the lower panel represents the 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 – +4°C; –1 –, –18°C; Heat — snap-heating. B) Graphical presentation of SARS-CoV-2 marker detection 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 shown in panel A. C) The sample solution allows detection of endogenous GAPDH. Suspension of MCF7 human cells, annotated as CellExtr 1.0 for 1x10^6 cells/ml and 0.01 for 1x10^4 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 1x10^6 cells/ml, 0.01 corresponds to 1x10^4 cells/ml. Quantification of the generated PCR product with ImageJ shown in the lower panel. Representative experiments out of a total of 3 (A, B) and 4 (C) are shown.



Supplementary Figure S1. The sample solution prevents microbial growth

A) 20 microL aliquots of saliva in sample solution or saliva alone were added to a growth media in duplicate wells of a 6-well plate. Media without added samples was used for control. The plate was incubated at 37°C for 72 h. Microbial growth was monitored under the microscope. Change of the color from red to yellow indicates microbial growth. Saliva collected in the sample solution containing 1 % Tween 20 and 40 % ethanol, at a 1:1 ratio, did not show microbial growth. B) Saliva proteins were separated by SDS-polyacrylamide gel electrophoresis. The pattern of proteins corresponds to patterns reported earlier. 10 and 20 microliters of saliva were separated in 10 % polyacrylamide gel, fixed and stained with Coomassie Brilliant Blue.

Discussion

Omitting nucleic acid purification may significantly facilitate PCR-based testing. However, the complexity of saliva 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 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; 16]. The composition of the sample solution described here promotes the preservation of genetic markers and also allows for the 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 enzymes in the PCR reaction (Figure 1). SDS, 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 the 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 benefits 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 targeted markers.

The storage of samples between collection and analysis is of importance for successful testing. Saliva contains enzymes and chemical entities that may affect the stability of markers. Approaches to preserving sample integrity include freezing, keeping at cold, and chemical or thermal stabilization [6; 8–9]. Storage at room temperature, i.e., 20°C and higher, is not recommended. We observed that the storage of samples at +4°C or −18°C for 24 h preserved markers (Figure 2). Storage at room temperature resulted in the degradation of markers already after 5 h. Snap-heating at +80°C for 5 min immediately following sample collection is aimed at denaturing enzymes in saliva [6, 8]. Stabilization of the genetic marker after snap-heating was observed (Figure 2A, B), although the efficiency of detection was lower as compared to storage at +4°C or −18°C.

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). We observed that the protocol described here provides for detecting copy numbers in the range described for protocols using the nucleic acid purification step. The detection range of the described protocol is in the range of 10 molecules per test reaction for all 3 tests described herein. It indicates that the performance of primers is of high importance for detection. This is similar to ranges reported for the Genmark ePlex and the Abbott RealTime SARS-CoV-2 tests, e.g., 10^2–10^3 copies per milliliter [19]. This protocol can be used in clinical trials to detect different genetic markers, including markers of SARS-CoV-2 and intracellular endogenous markers.

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

The protocol describes the collection of saliva in a solution containing Tween 20 and ethanol, storage conditions (+4°C or frozen), and shows compatibility with PCR and LAMP methods. Our report also describes 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 the implementation of the protocol reported herein. The robustness and simplicity of this protocol are of advantage for its clinical use.

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.

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.

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