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Pre-analytical properties of different respiratory viruses for PCR-based detection: Comparative analysis of sampling devices and sample stabilization solutions

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ABSTRACT

After the decline of the COVID-19 pandemic, health systems were challenged by the simultaneous prevalence of different respiratory viruses causing a wide overlap in symptoms. This increased the demand for multi-virus diagnostic tests which require suitable pre-analytical workflow solutions in order to receive valid diagnostic results. In this context, the effects of specimen storage duration and temperature on the RNA/DNA copy number stability of influenza A/B, RSV A/B, SARS-CoV-2 and adenovirus were examined for four commercially available transport swab systems and saliva collection devices. The respiratory viruses were more stable in the saliva collection devices than in the transport swab systems when stored at RT or 37 °C for up to 96 h. Moreover, no differences between viral nucleic acid stability of enveloped and non-enveloped viruses were observed. The infectivity of all enveloped viruses could be inactivated by the saliva collection device from PreAnalytiX. The Norgen saliva device completely inactivated influenza A/B, while RSV A/B were partially inactivated. The non-enveloped adenovirus was inactivated by a reduction factor of 10E+ 4 in both saliva collection devices. All respiratory viruses remained infectious in the transport swab systems. Two possible transport medium additives were tested which inactivated or strongly reduced viral replication of tested enveloped viruses but had no effect on the non-enveloped adenovirus. Finally the implementation of multi-target detection procedures involving a direct amplification approach was successfully tested by spike-in of all enveloped viruses simultaneously into transport swab systems. This fast and reproducible setup presents a valuable solution for future implementations in multi-virus testing strategies.

1. Introduction

The COVID-19 pandemic has clearly demonstrated the importance of diagnostic testing for respiratory viruses. Although the COVID-19 pandemic has officially ended, SARS-CoV-2 together with other respiratory viruses will continue to play a major role in endemic cold waves each year. Since the symptoms of viral respiratory infections are not specific for a particular virus, there is an increasing need for testing of the most relevant respiratory viruses, as treatment and management of these infections, as well as public health measures, differ depending on the virus species [1]. This goes hand-in-hand with the need for sample collection and stabilization devices that are appropriate for the different virus types.

Human respiratory viruses may have different pre-analytical properties based on differences in their structure and molecular composition. For example, Influenza, RSV and SARS-CoV-2 belong to the enveloped group, while adenoviruses belong to the non-enveloped viruses. Like all other viruses of its group, influenza viruses are negative-sense single-stranded RNA viruses, which are vulnerable to environmental conditions such as humidity and temperature. They are sensitive towards lipid solvents and detergents [1,2]. RSV is also a single-stranded negative RNA virus. This species is subdivided into the two groups A and B and

Abbreviations: A549, human lung carcinoma epithelial cells; BE, Artificial body excretion; CPE, Cytopathic effect; EMA, European Agency for the Evaluation of Medicinal Products; MDCK, Madin-Darby Canine Kidney; UTM, universal transport media; VeroE6, African green monkey kidney epithelial cells; VTM, viral transport media; VPM, viral preservative medium.

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Table 1
Specimen collection, transport and storage devices and saliva collection devices and respective manufacturers' claims.

Device	Abbreviation	Storage Condition	Regulatory Status during study 2022
Copan UTM-RT® Universal Transport Medium 359 C + Single Regular Size Nylon® Flocked Swab	UTM	INTENDED USE UTM® is an FDA cleared collection and transport system suitable for collection, transport, preservation and long-term freeze storage of clinical specimens containing viruses, including COVID-19, chlamydia, mycoplasma or urea plasma organisms. The transport medium comes in a plastic, screw cap tube and maintains organism viability for 48 h at room and refrigerated temperature.[8]	EU: CE IVD Outside EU: FDA cleared
IMPROVIRAL™ Viral Preservative Medium (8110111) + ImproSwab® (550040 A)	VPM	Widely used for the collection, preservation and transportation of nasopharyngeal pathogen specimens such as influenza, pneumonia, avian influenza, hand-foot-mouth disease, measles and other. Storage and transport under 0-8 °C and no more than 30 days at room temperature. VPM is intended for the collection and transportation of clinical specimens containing viruses from the collection site to the testing laboratory. It maintains the viability of the virus and integrity of the sample.	EU: CE IVD
PAXgene Saliva Collector (769040) – (PreAnalytiX GmbH, Switzerland)	PreAnalytiX	Saliva collected and stabilized with the PAXgene Saliva Collector can be stored for DNA isolation for at least 24 months at temperatures up to 25 °C (study ongoing). In addition, PAXgene Saliva can be frozen long term at − 20 or − 80 °C when transferred into a suitable cryovial. SARS-CoV-2-derived RNA copy numbers are stabilized in saliva collected into PAXgene Saliva Collector for at least 4 days (96 h) at 20 °C[9].	MBA (for molecular biology applications) world wide
Saliva RNA Collection and Preservation Device Dx (53810) – (Norgen Biotek Corp. Canada)	Norgen	Once collected, saliva RNA is stable for up to 2 months when kept tightly sealed and stored at room temperature[10].	EU: CE IVD Outside EU: authorized for diagnostic use in Canada by Health Canada

can be inactivated by a 5 min treatment at 55 °C due to its thermolability [2]. The human adenoviruses are non-enveloped double-stranded DNA viruses and have a higher resistance against environmental conditions compared to the other viruses mentioned above. They survive freeze and thaw cycles with only minimal loss of viral copy numbers [3].

Only a few studies have so far examined the important pre-analytical phase including collection, handling and storage conditions of specimens from patients with respiratory infections [4–6]. In a study on pre-analytical factors affecting the detection of SARS-CoV-2 [7], marked differences in pre-analytical stability were found for swap or saliva sampling devices as well as different transport media depending on simulated transport duration and temperature.

Currently, different cotton and nylon swabs are used in combination with different media such as viral transport media (VTM) or universal transport media (UTM) to obtain nasopharyngeal samples. These media are suitable for cultivation of microorganisms for further analysis. Saliva collection devices can have inactivating capacities and can enable home collection. Several studies have analyzed the performance of saliva and nasopharyngeal specimens for RT-qPCR, and showed that saliva based nucleic acid amplification tests (NAAT) could be an attractive alternative to nasopharyngeal swab based NAAT due to their similar diagnostic accuracy [5,6].

In order to address the need of diagnostic test for multiple respiratory viruses we extend in this study our previous study [7] on pre-analytical factors related to SARS-CoV-2 to other respiratory virus types, namely Influenza virus A and B, human respiratory syncytial virus A and B as well as human Adenovirus 2.

2. Material & methods

2.1. Saliva collection devices and transport swab systems

The used saliva and swab systems, the recommended storage conditions and claims towards inactivation properties are listed in Table 1. Information on the devices was cited from the suppliers' homepages including relevant instructions for use.

2.2. Cell culture

African green monkey kidney epithelial cells (VeroE6) were from Biomedica (VC-FTV6, Vienna, Austria) and were grown in Minimal Essential Medium (MEM) containing Earle's Salts and L-Glutamine (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 2% fetal calf serum (FCS) (Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (PenStrep) (Thermo Fisher Scientific). Madin-Darby Canine Kidney (MDCK – NBL-2) cells were from CLS (602280) (Cell Lines Service GmbH, Eppelheim, Germany) and were grown in DMEM Ham's F12 medium (Cell Lines Service GmbH), supplemented with 1% PenStrep. A549, human lung carcinoma epithelial cells, were from Cell Bank Graz (CLS 300114) and were grown in DMEM Ham's F12 medium supplemented with 10% FCS and 1% PenStrep.

2.3. Respiratory viruses and their propagation

All experimental procedures with viruses were performed in a BSL-3 laboratory to ensure comparable experimental conditions for all viruses tested. All viruses and cell lines used are summarized in Table 2. Human 2019-nCoV Isolate (Ref-SKU: 026 V-03883, purchased from Charité, Berlin, Germany) was propagated in VeroE6 cells and virus titer was determined by a focus forming assay as described previously [7]. Human Adenovirus 2 strain INMI (REF-SKU: 009 V-03848, EVAg, Marseille, France) was propagated in VeroE6 cells at 37 °C and 5% CO_2 for 72 h. Influenza Virus Infectious B/Washington/02/2019 (Washington B) was from NIBSC, Ridge, UK. Human respiratory syncytial virus A, Strain: RSV-A/EMC58 (RSV A) (Ref-SKU: 011 V-01985) and Human respiratory syncytial virus B, Strain: RSV-B/EMC88 (RSV B) (Ref-SKU: 011 V-01981) were from Erasmus MC via EVAg, Marseille, France. These three viruses were propagated in A549 cells at 37 °C and 5% CO_2 for 72 h. Prior to harvesting, cells were lysed by a freeze and thaw cycle, followed by a centrifugation step (5 min, 1000 x g at RT) and the supernatant was filtered with 0.2 µm syringe filters (Thermo Fisher Scientific). Influenza Virus Infectious A/Guandong-Maonan/SWL1536/2019 (H1N1) (ord.No.19/294, NIBSC, Ridge, UK) was propagated in MDCK cells treated with 0.2 µg/ml TPCK-Trypsin (Thermo Fisher Scientific, Waltham, USA) at 35 °C and 10% CO_2 for 96 h. Prior to harvesting, cells were lysed by up to 3 freeze and thaw cycles, followed by centrifugation and filtration as described above. All virus stocks were stored at − 80 °C until further use.

2.4. Virus isolation and quantification via RT-qPCR

All RT-qPCRs were performed on a Rotor Gene® Q cycler (QIAGEN

Table 2
Viruses and cell cultivation.

Virus	Abbreviation	Purchased from	Cell cultivation
Human 2019-nCoV Isolate ex China Strain: BavPat1/2020 (Ref-SKU: 026 V-03883)	SARS-CoV-2	Charité , Berlin, Germany	Vero E6 (Biomedica, VC-FTV6)
Human Adenovirus 2 strain INMI (REF-SKU: 009 V-03848)	Adenovirus	EVAg, Marseille, France	Vero E6 (Biomedica, VC-FTV6)
Influenza Virus Infectious A/Guandong-Maonan/SWL1536/2019 (H1N1) (ord.No.19/294)	Influenza H1N1	NIBSC	MDCK (NBL-2) (CLS, Ord. no. 602280)
Influenza Virus Infectious B/Washington/02/2019	Influenza Washington B	NIBSC	A549 (CLS, Ord. no. 300114)
Human respiratory syncytial virus A, Strain: RSV-A/EMC58 (Ref-SKU: 011 V-01985)	RSV A	EVAg, Marseille, France via Erasmus MC	A549 (CLS, Ord. no. 300114)
Human respiratory syncytial virus B, Strain:RSV-B/EMC88 (Ref-SKU: 011 V-01981)	RSV B	EVAg, Marseille, France via Erasmus MC	A549 (CLS, Ord. no. 300114)

GmbH, Hilden, Germany). The applied RNA isolation kits, RT-qPCR kits and virus standards are summarized in Table 3. Viral RNA from Washington B, RSV A and B and SARS-CoV-2 was isolated using QIAamp® Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions for use. RNA samples were eluted in 40 µl ultra-pure water. 5 µl of the eluate from samples spiked with Washington B were used for RT-qPCR using artus Infl./H1 LC/RG RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). RT^2 First Strand Kit (QIAGEN, Hilden, Germany) was used for cDNA synthesis of 10 µl isolated RSV A/B RNA according to manufacturer's instructions and 10 µl of cDNA were introduced into tests performed with the Microbial DNA qPCR Assay for RSV. 5 µl of the eluate from samples spiked with SARS-CoV-2 were used for RT-qPCR using N2 primers (Eurofins Genomics, Ebersberg, Germany) and a probe set of the 2019-Novel Coronavirus (2019-nCoV) Real-time rRT-qPCR Panel in combination with the QuantiTect® Multiplex RT-qPCR Kit (QIAGEN GmbH, Hilden, Germany). Viral RNA from H1N1 was extracted from the lysate of MDCK cells using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions and 5 µl were used for RT-qPCR using Artus Infl/H1 LC/RG RT-PCR Kit (QIAGEN GmbH, Hilden, Germany). Viral DNA from adenovirus was isolated using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions and 10 µl of eluted DNA was taken to perform RT-qPCR (Artus HAdV RG PCR Kit (QIAGEN GmbH, Hilden, Germany).

To determine viral copy numbers, commercially available standards from ATCC (summarized in Table 3) were used for generation of standard curves in each RT-qPCR run. The resulting Ct-values were plotted against ln[copy numbers] and the equation obtained from a simple linear regression analysis was used to calculate the copy numbers from the Ct-values.

$$\text{Adenovirus} : y = -1.36x + 36,9$$

$$\text{H1N1} : y = -1.84x + 41,8$$

$$\text{Washington B} : y = -1.33x + 36,4$$

$$\text{RSV A} : y = -1.38x + 36.7$$

$$\text{RSV B} : y = -1.44x + 40,2$$

$$\text{SARS} - \text{CoV} - 2 : \text{N2} : y = -1.51 + 38.4$$

The cut-offs were set to 38.4 for SARS-CoV-2 N2, 38.4 for adenovirus, 41.8 for H1N1, 36.1 for Washington B, 36.7 for RSV A, 40.2 for RSV B, respectively. Ct-values exceeding the cut-off were set to 1 for graphical representation.

2.5. Stability test setup of respiratory viruses

Room temperature (RT) ranged from 18 °C to 25 °C as defined by the ISO standard ISO 4307:2021 "Molecular in vitro diagnostic examinations – Specifications for pre-examination processes for saliva – Isolated human DNA". The actual range measured during our studies was 23 °C – 24 °C. The experimental conditions were previously established in [7]. The two most suitable transport swab systems and saliva collection devices were selected from this study (Table 1) to compare adenovirus DNA and H1N1, Washington B, and RSV A/B RNA stability at different storage conditions.

For the swab systems, artificial oral fluid matrix (BE) was prepared by mixing 2.5 mg/ml BSA (Carl Roth GmbH, Karlsruhe, Germany), 3.5 mg/ml tryptone (Becton Dickinson, Le Pont de Claix, France) and 0.8 mg/ml mucin (Merck KGaA) [11]. This medium was spiked with 100,000 (= high spike-in), 10,000 (= medium spike-in) and 1000 (= low spike-in) copies of each virus separately per swab device. 100 µl of spiked BE were applied onto each swab which was directly transferred into the respective transport medium. Spiked transport swab systems were stored at RT and at 37 °C for zero h (t0), 24 h (t24) and 96 h (t96), respectively. At each time point, transport media of swab samples were mixed by pipetting, and a sample was taken for viral DNA or RNA isolation and quantification. Every swab transport system was examined for virus stabilization in two independent experiments with three technical replicates each, only adenovirus stabilization was tested in two independent experiments once with three and once with six technical replicates.Viral nucleic acids were isolated and quantified via RT-qPCR as described above. Detected viral copy numbers of each respiratory virus were extrapolated to the total copy numbers per device according to the equations listed above.

The collection of saliva from self-reporting healthy volunteers was approved by the research ethics committee of the Medical University Graz (approval number EK 32–666). After obtaining informed consent, saliva from three or four healthy donors was pooled and spiked with the same three virus copy numbers as for the swab systems. Two ml of spiked saliva was added to 1 ml of each saliva stabilization solution. Spiked saliva collection devices were stored at the same temperatures and duration as the swab samples (RT, 37 °C). At each time point, devices were vortexed and replicates aliquoted from each device. Two biological replicates with three technical replicated each were examined for each tested condition. Viral nucleic acids were isolated, quantified by RT-qPCR and extrapolated to the total copy numbers per device.

2.6. Testing viral inactivation properties

The infectivity inactivation properties of the tested saliva and swab systems for the five tested human respiratory viruses were examined in in-vitro infection assays. For each virus, cells were seeded in 48-well cell culture plates (30,000 cells/well) and cultivated overnight to reach approximately 80% confluence on the day of infection.

The reagent from each saliva and swab systems was spiked with each examined virus and diluted in serum free-medium, to avoid possible cell cytotoxic effects by the preservation solutions in the devices. After 1 h incubation (at 37 °C and 5% CO_2, except for H1N1 at 35 °C and 10% CO_2) cells were washed once with medium and incubated in fresh culture medium for 72 h or 96 h, respectively. Virus infection and

Table 3
RNA / DNA isolation kits and RT-qPCR Kits.

Virus	RNA Isolation Kit	DNA Isolation Kit	RT-qPCR Kit	Standards
SARS-CoV-2	QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany)	-	● QuantiTect Multiplex RT-qPCR Kit (QIAGEN GmbH, Hilden, Germany) ● N2 primer Panel (Quelle) 2019-nCoV_N2-F 2019-nCoV_N2 Forward Primer 5′- TTA CAA ACA TTG GCC GCA AA – 3′ 2019-nCoV_N2-R 2019-nCoV_N2 Reverse Primer 5′- GCG CGA CAT TCC GAA GAA – 3′ 2019-nCoV_N2-P 2019-nCoV_N2 Probe 5′- FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3	ATCC VR-1986D genomic RNA from 2019 Novel Coronavirus Lot: 70035624 (ATCC, Manassas, US)
Adenovirus	-	QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany)	Artus HAdV RG PCR Kit (QIAGEN GmbH, Hilden, Germany)	Available in PCR Kit
Influenza H1N1	RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany)	-	Artus Infl./H1 LC/RG RT-PCR Kit (QIAGEN GmbH, Hilden, Germany)	ATCC VR-1894DQ Quantitative Genomic RNA from Influenza A virus strain A/California/07/2009 pdm09 (H1N1), Lot: 70046261 (ATCC, Manassas, US)
Influenza Washington B	QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany)	-	Artus Infl./H1 LC/RG RT-PCR Kit (QIAGEN GmbH, Hilden, Germany)	ATCC VR-1804DQ Quantitative Genomic RNA from Influenza B virus strain B/Florida/4/2006, Lot: 70044182 (ATCC, Manassas, US)
RSV A / RSV B	QIAamp Viral RNA Mini Kit (QIAGEN GmbH, Hilden, Germany)	cDNA Kit: RT^2 First Strand Kit (QIAGEN GmbH, Hilden, Germany)	Microbial DNA qPCR Assay BPID00572AR (QIAGEN GmbH, Hilden, Germany)	RSV A: ATCC VR-1540DQ Quantitative Genomic RNA from Human respiratory syncytial virus strain: A2, Lot: 70043334; RSV B: ATCC VR-955DQ Quantitative Genomic RNA from Human respiratory syncytial virus strain 9320, Lot: 70030267 (ATCC, Manassas, US)
Multiplex Testing (SARS-CoV-2, Influenza A, Influenza B, RSV A/B)	QIAprep & amp Viral RNA UM Kit (QIAGEN GmbH, Hilden, Germany)	-	Multitarget detection assay for respiratory viruses (Biomers, Ulm, Germany)	

replication was assessed based on cytopathic effects (CPE) of VeroE6 and A549 cells and was documented after 72 h post infection via bright field microscopy. Additionally, 140 µl cell culture supernatant or lysate (MDCK cells infected with H1N1) was collected. Viral DNA or RNA was isolated as described before and was quantified by RT-qPCR.

Additionally, two additives, QIAprep&amp Transport Medium Additive-D and -P (QIAGEN GmbH, Hilden, Germany; TMA-D, TMA-P) were tested for their inactivating effect on the different viruses infectivity by the same in vitro experimental procedures as described above. Different concentrations of TMA-D or TMA-P were added to the respective cell culture medium. Each virus, including SARS-CoV-2, was separately spiked in the medium to the same concentration as for saliva and swab systems and incubated on the cells for 1 h (37 °C, 5% CO_2, except H1N1 35 °C and 10% CO_2). Thereafter, the cells were washed once with medium and incubated in fresh culture medium 72 h or 96 h post infection until viral replication in the supernatant or lysate was measured by RT-qPCR.

2.7. Multiplex testing of four different viruses

The four respiratory viruses influenza A H1N1, influenza Washington B, RSV A and SARS-CoV-2 were spiked simultaneously in BE in combination with one transport swab system. Three different virus concentrations were spiked-in according to the methods described above. The QIAprep&amp kit (QIAGEN GmbH, Hilden, Germany) was combined with the Multitarget Detection Assay specific for SARS-CoV-2, influenza A/B and human RSV A/B (Biomers.net GmbH, Ulm, Germany). 18 µl of the spiked-in samples were added to 6 µl of buffer AB and heat inactivated at 95 °C for five minutes. 2 µl of preparation buffer and 8 µl inactivated samples were mixed and 15 µl of PCR master mix were added. RT-qPCR was performed on a Rotor Gene® Q cycler (QIAGEN GmbH, Hilden, Germany).

3. Results

Four saliva and swab systems (Table 1) were selected based on results obtained in a widespread screening approach on SARS-CoV-2 RNA stability [7]. These were used to test the stability of viral nucleic acids of adenovirus 2, influenza A H1N1, influenza Washington B, and RSV A and B at different storage durations and temperature conditions as well as their ability to inactivate these respiratory viruses.

3.1. Influenza A H1N1 virus is stable in saliva collection devices and can be inactivated

As shown in Fig. 1, the copy number of influenza A H1N1 RNA found in both swab systems (UTM from Copan, VPM from Improve Medical) slightly decreased after 24 h and 96 h for all spike-in concentrations and increased at 37 °C. In addition, in one experimental series using VPM, the RNA detection failed completely for the lowest spike-in concentration at RT, and we found one dropout at 37 °C in UTM and VPM with the middle range spike-in concentration. Both saliva devices (from PreAnalytiX and Norgen) were able to stabilize viral RNA of influenza A H1N1 in saliva over 96 h at RT and 37 °C, although at RT the detection of viral RNA from samples stored in the Norgen device appeared more variable.

Viral replication could not be observed in cell culture-based infection assays after specimen storage of 96 h in the saliva devices from PreAnalytiX and Norgen. In contrast, influenza A H1N1 was able to replicate when spiked into the swab systems UTM and VPM, comparable to medium-treated positive control.

TMA-D and TMA-P could decrease the viral replication of influenza A H1N1 in the cells in a concentration-dependent manner after 96 h of specimen storage. However, for TMA-D, two out of three in one experimental series showed viral replication even at the highest TMA-D concentration. TMA-P reduced viral replication of influenza A H1N1 with a maximum reduction of log10 E + 1.92.

3.2. Influenza virus Washington B is stable in saliva collection devices and can be inactivated by them as well as by TMA-D and TMA-P

Decrease of viral RNA copy numbers of influenza Washington B was observed at all spike-in concentrations after 24 h and 96 h compared to timepoint 0 h when stored at RT or 37 °C in UTM and VPM for one experimental series (Fig. 2). In the second series, a decrease of viral RNA copies was detected only after 96 h under both temperature conditions. The lowest spike-in concentration showed the most pronounced reduction of viral RNA copy number. Viral RNA became undetectable after 24 h or 96 h in several replicates. In contrast, no alteration of viral RNA copy numbers from influenza Washington B was detectable in the PreAnalytiX and Norgen devices stored at RT or at 37 °C for up to 96 h for all three spike-in concentrations. The actual measured spike-in concentrations were higher than the calculated spike-in rate in one experimental series with the PreAnalytiX device, but viral copies were maintained at that high level.

Furthermore, no viral replication was measured in the supernatant of the cells after 72 h when influenza Washington B was treated with the PreAnalytiX device. The virus replication could also be inhibited by the Norgen device reagent, however, with one out of three replicates from one experimental series showing minimal viral replication after 72 h.

Less virus RNA compared to the positive control (10E+1.28 for UTM and 10E+0.79 for VPM) was recovered from UTM and VPM samples. Influenza Washington B could be re-cultured in the cell culture medium (positive control) and no infectivity inhibition effect could be observed.

Similarly, viral replication could be measured in cultures for some samples when influenza Washington B was treated with the two medium additives. The mean $\log_{10}$ reduction of 10E+ 5.67 and 10E+ 4.47 caused by the media with the highest TMA-D/TMA-P concentration indicated a viral inhibition while some replicates were inhibited completely and viral replication for others was just reduced.

3.3. RSV A is stable and cannot be inactivated completely under some tested conditions

In general, the theoretical spike-in concentrations of all dilutions used for the swab transport systems did not reflect the measured RT-qPCR results from samples processed immediately after spike-in at timepoint 0 (Fig. 3). In both experiments, viral copy numbers collected from UTM and VPM samples stored at RT and 37 °C samples were variable and no specific trend was recognised.

Additionally, RSV A copy numbers at low spike-in concentrations showed a higher stability in the PreAnalytiX saliva collection device than in the Norgen device, at both storage temperatures (Fig. 3 A). For high and middle spike-in concentrations, both devices showed stabilization of RSV A RNA.

PreAnalytiX and Norgen devices were able to inhibit virus replication of RSV A in A549 cells in all three replicates of one experimental series and in two out of three replicates in the other series. VPM and

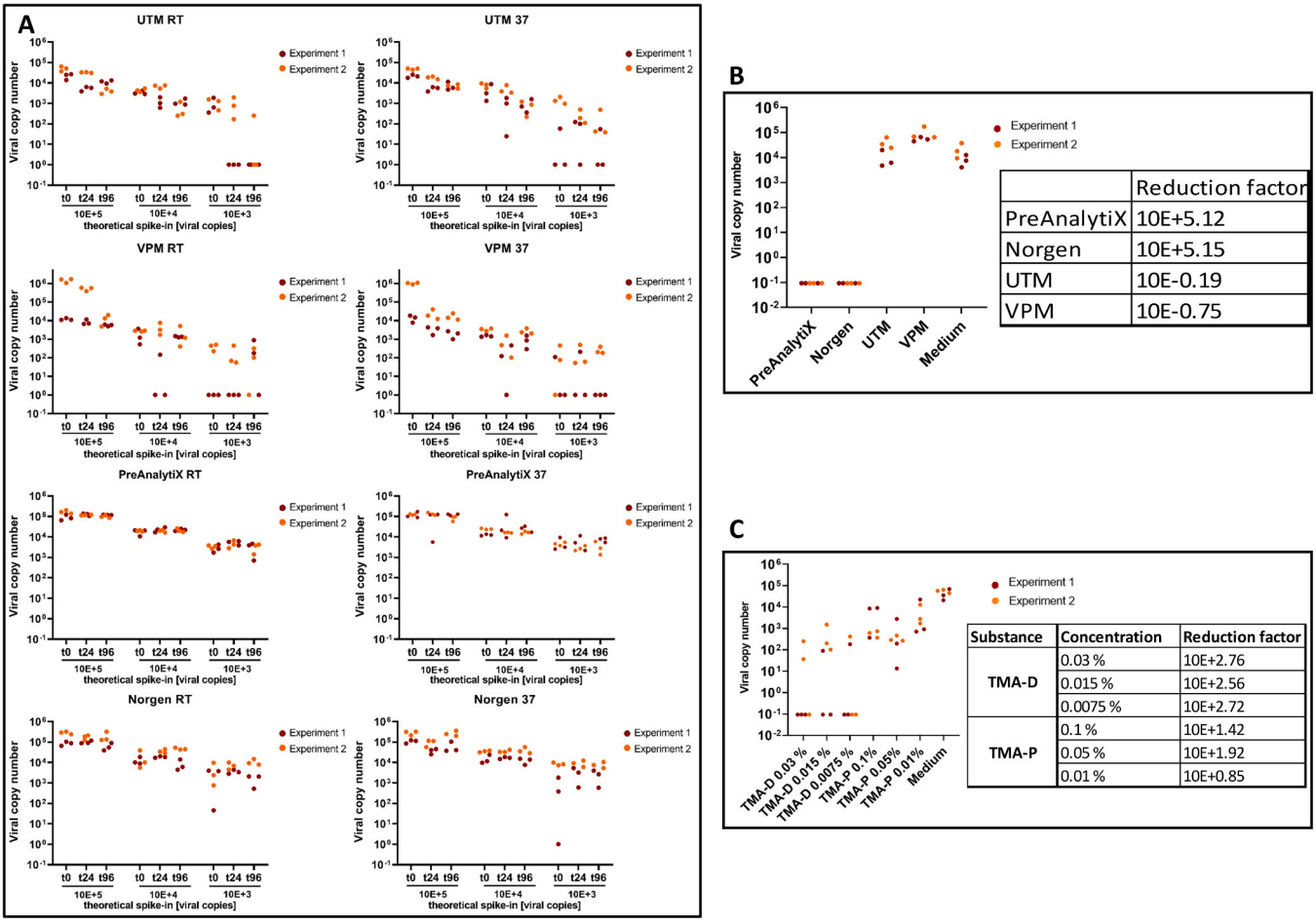


Fig. 1. (A) Stabilization of influenza A H1N1 RNA by swab systems and saliva collection devices stored for up to 96 h at RT and 37 °C, respectively. Data shown refer to two independent experimental series with three replicates each. (B and C) Infectivity of influenza A H1N1 in the tested swab systems, saliva collection devices or by cultivation with respective medium additives in MDCK cells after 96 h determined by RT-qPCR. Cell culture medium (Medium) spiked with the same viral load served as positive control. Log10 reduction factors were calculated in relation to the positive control. Data shown refer to two independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per system or device.

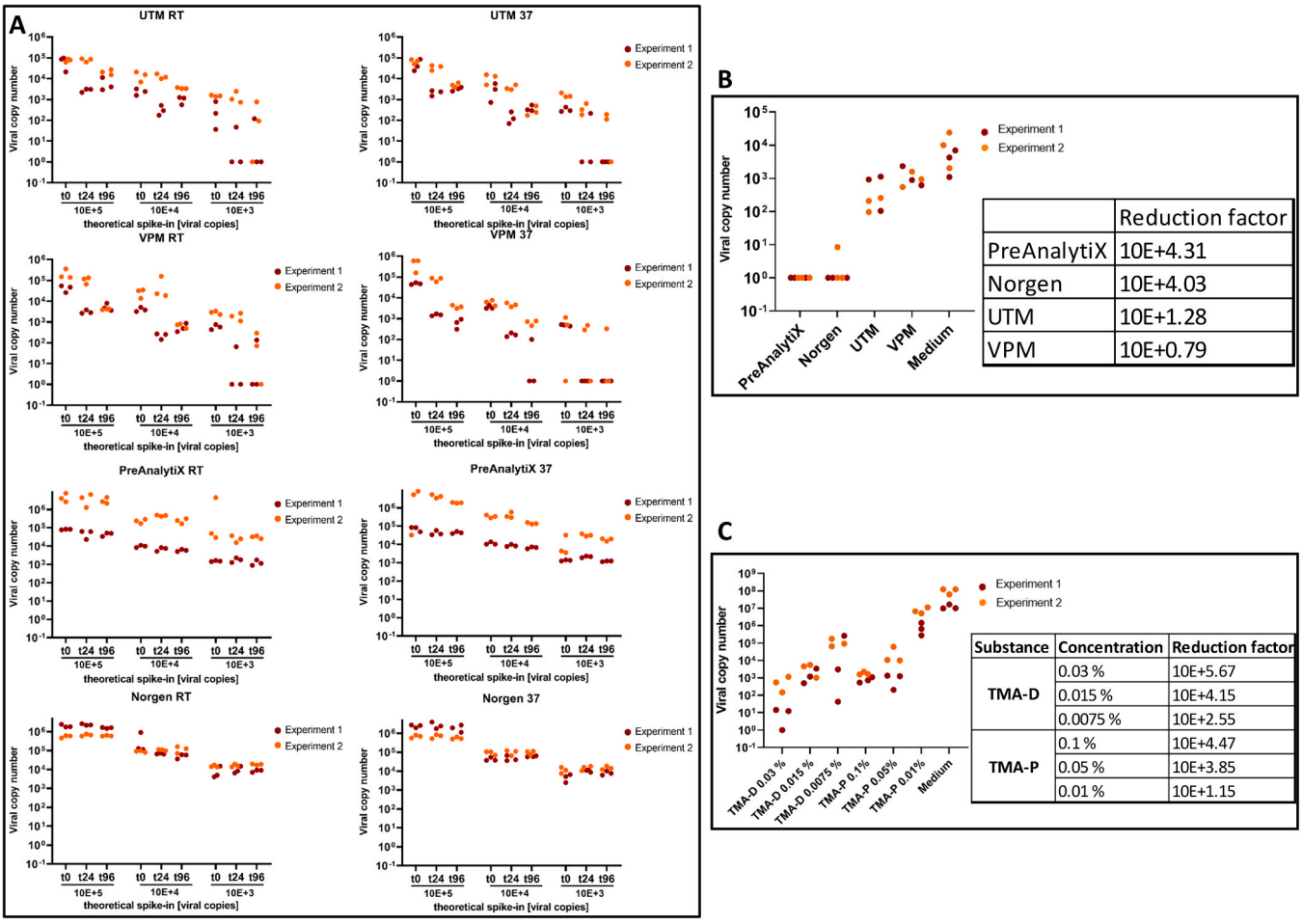


Fig. 2. (A) Stabilization of influenza Washington B RNA by swab systems and saliva collection devices at RT and 37 °C for up to 96 h. Data shown refer to two independent experimental series with three replicates each (Experiment 1 and 2). (B and C) Infectivity of influenza Washington B in the tested swab systems, saliva collection devices (B) or with respective medium additives (C), by cultivation in A549 cells for 72 h, determined by RT-qPCR. Cell culture medium (Medium) spiked with the same viral load as used for spiking the collection devices served as positive control. Log10 reduction factors were calculated in relation to this positive control. Data shown refer to two independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per swab system or saliva device.

UTM showed a reduction of 10E+ 2 compared to the positive control, showing low viral replication rate.

The highest concentration of TMA-D in the added media led to a complete inhibition of RSV A infectivity in one experimental series and in two out of three replicates in the other one, while all lower concentrations of TMA-D as well all concentrations of TMA-P failed to inhibit the viral replication in the cells.

3.4. RSV B is stabilized in saliva collection devices and still infectious under most tested conditions

As with RSV A, the theoretical spike-in concentrations of RSV B in the swab system UTM and the actual measured viral copy numbers did not align well for either independent experiment (Fig. 4). Viral RNA copy numbers detected in one experiment with the UTM swab were stable at RT and 37 °C. However, in the second experiment, two replicates of the lowest spike-in concentration at 37 °C as well as one middle spike-in replicate at RT could not be detected already at t0.

RSV B RNA copy numbers were maintained in VPM at RT as well as at 37 °C at high spike-in concentrations only. Independent of storage conditions, middle and low spike-in concentrations showed variable viral RNA copy number with a high RT-qPCR dropout rate.

RSV B viral RNA was stable in the PreAnalytiX and Norgen devices at both storage temperatures with minor fluctuations (Fig. 4A). The PreAnalytiX saliva device completely inactivated RSV B, and two out of three replicates in each experimental series incubated with the Norgen device showed inactivation as well (Fig. 4B). UTM and VPM reduced the viral replication of RSV B in A549 cells compared to the control by a factor of 10E+ 2.3 and 10E+ 2.4, respectively. Additionally, the medium with the highest concentration of TMA-P was able to inhibit viral replication compared to the positive control in all three replicates in both experimental series. The second highest TMA-P concentration led to inhibition of RSV B infectivity in five out of six replicates (Fig. 4C). For the two highest TMA-D concentrations, a complete inhibition of viral replication was only observed in the second experimental series but failed in two replicates in the first experimental series.

3.5. Adenovirus 2 is more stable in saliva collection devices and remains infectious

A trend of reduced adenovirus DNA copy numbers in the UTM swab system could be observed after 24 h and 96 h at RT for the low spike-in concentration (Fig. 5A). At 37 °C, a reduction in viral copy numbers was detected for high and middle spike-in numbers as well. Moreover, the recovered DNA was below the limit of detection of the applied RT-qPCR test in several samples stored at either RT and at 37 °C. Furthermore, in one of the two experimental series, it is evident that the amount of viral DNA was increased after 96 h. In the VPM swab system, viral DNA was

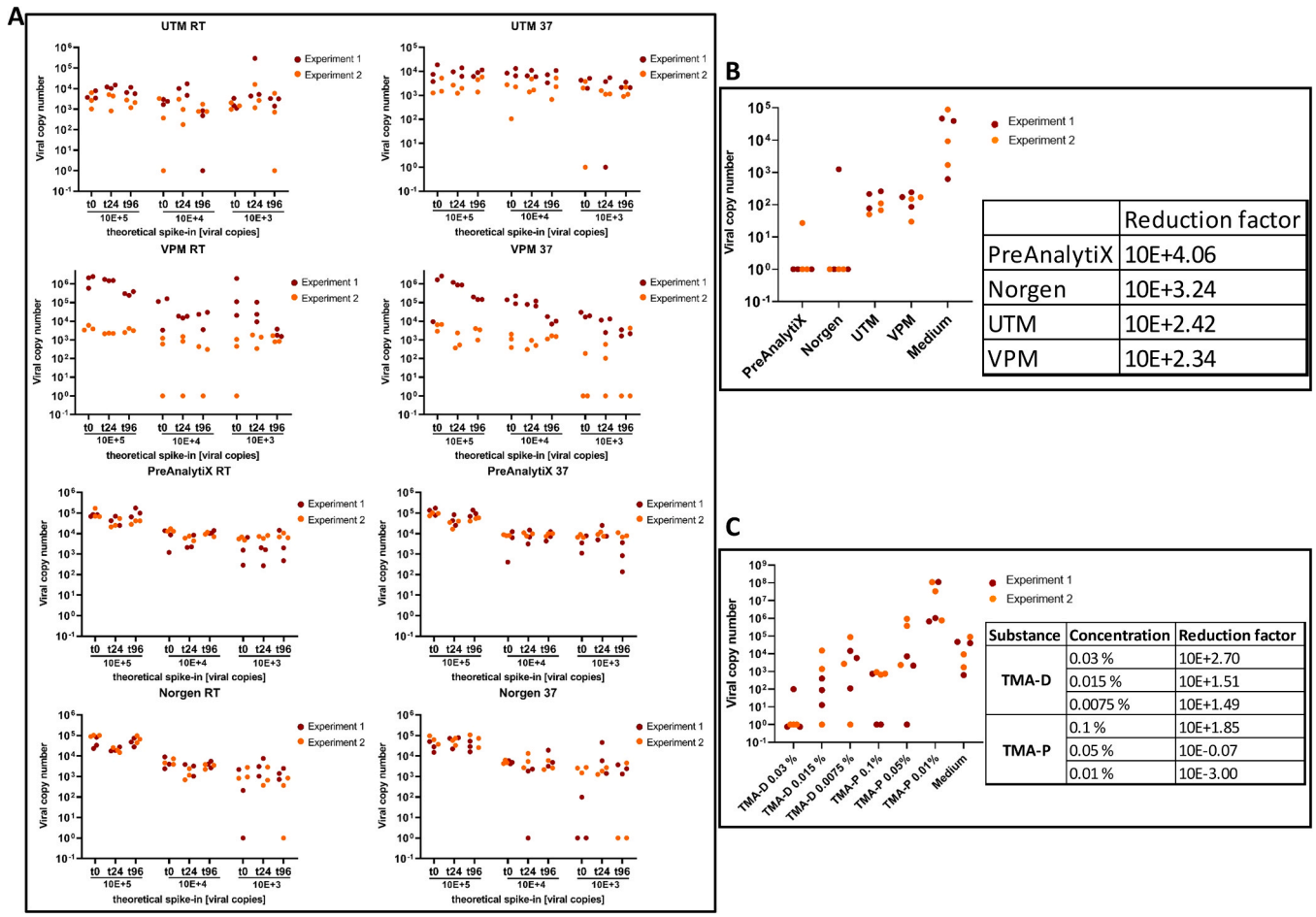


Fig. 3. (A) Stabilization of RSV A RNA by swab systems and saliva collection devices at RT and 37 °C during storage of up to 96 h. Data shown refer to two independent experimental series with three replicates each (Experiment 1 and 2). (B and C) Infectivity of RSV A in the tested original swab systems, saliva collection devices (B) or with respective additives (C), by cultivation in A549 cells for 72 h, determined by RT-qPCR. Cell culture medium (Medium) spiked with the same viral load as used for spiking the collection devices served as positive control. Log10 reduction factors were calculated in relation to this positive control. Data shown refer to two independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per system or device.

stabilized only at the highest spike-in at both storage temperatures, while middle/low spike-in concentration could not be stabilized as these samples showed high variation in viral copy numbers already at time point zero as well as a high number of dropouts at later timepoints.

In contrast to the swab systems, adenovirus DNA levels remained constant over 96 h in the saliva collection device PreAnalytiX at RT and at 37 °C (Fig. 5A). In comparison, Norgen showed a high fluctuation of the detected viral DNA at both storage temperatures for the lowest spike-in concentration starting after 24 h.

The adenovirus infectivity inactivation properties of the saliva and swab systems and the two additives were tested in cell culture-based infection assays after 72 h of incubation. As shown in Fig. 5B, the swab systems UTM and VPM were not able to inactivate adenoviruses, indicated by the viral replication observed in the infection assay. Both saliva collection devices reduced the amount of the infectious virus compared to the positive medium control and were able to inhibit the adenovirus replication by a factor of 10E+ 4. The calculated infectivity reduction factor of 10E+ 4 when storing adenovirus in the PreAnalytiX device is described by EMA as an effect on viral removal and inactivation, even though it does not guarantee complete virus inactivation [12].

In contrast to the enveloped viruses tested, the adenovirus 2 was not inactivated by the medium additives TMA-D and TMA-P.

3.6. SARS-CoV-2 is inactivated by both transport medium additives

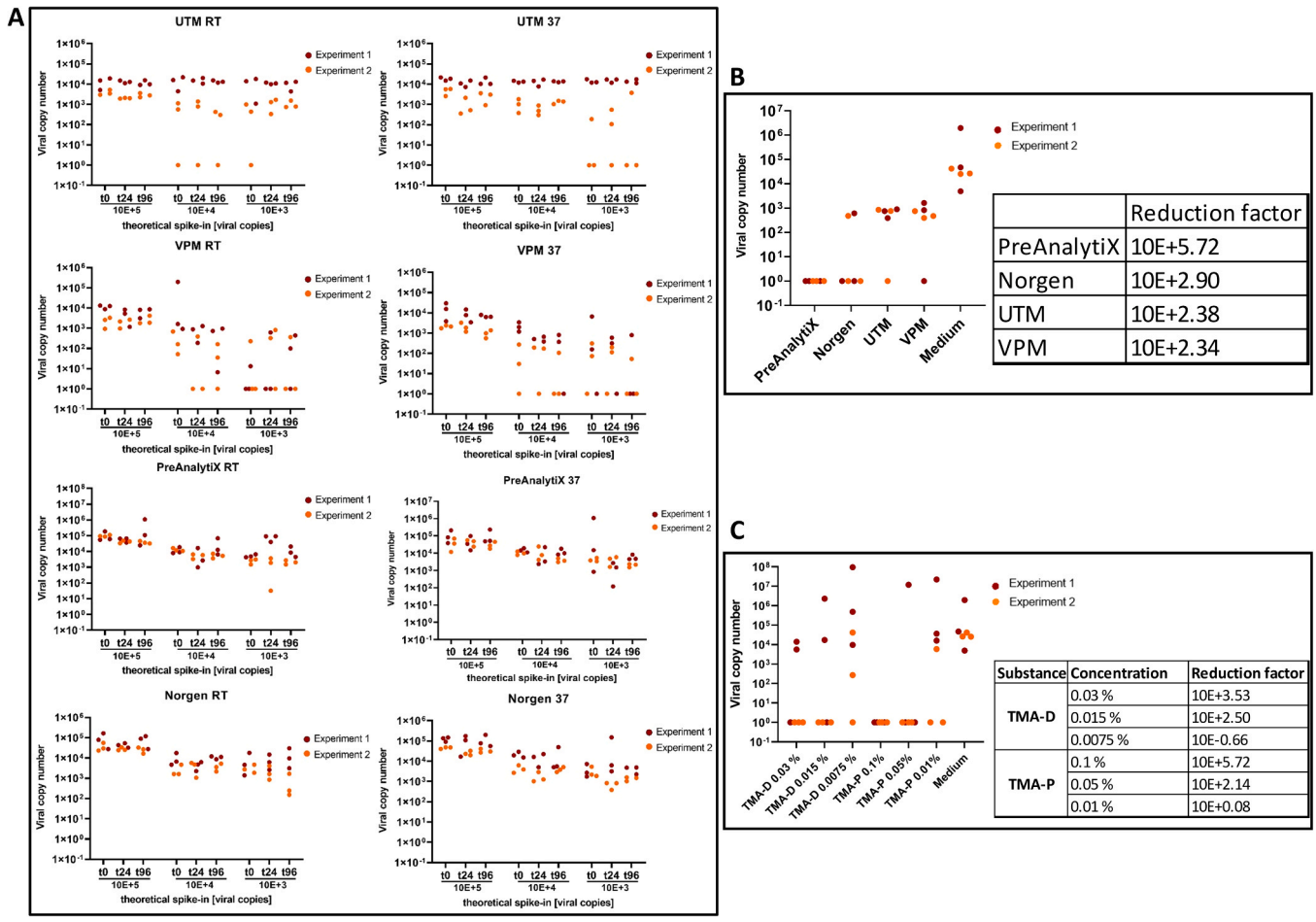
Inactivation properties were recently reported of different saliva collection devices, including the two devices tested here, which were not seen in swab systems [7]. As a concept, inactivating additives could be added to viral transport media to increase safety measures for personnel working at sample collection sites and laboratories. Two medium additives were therefore tested in pre-analytical workflows (Fig. 6). SARS-CoV-2 infectivity could be inactivated by all tested concentrations of TMA-D and in a dose-dependent manner by TMA-P. For TMA-P, only the two highest concentrations were able to prevent cell infection.

3.7. Four respiratory viruses can be detected simultaneously

SARS-CoV-2, influenza A H1N1, influenza Washington B and human RSV A were spiked-in in UTM and VPM at three different concentrations of each virus (Fig. 7). Virus spike-in concentrations were adjusted for each virus to yield similar Ct-values. The spike-in concentrations and triplicates showed repeatability in both experiments.

4. Discussion

In this study, the influence of pre-analytical variables like temperature (RT and 37 °C), storage/transport durations (up to 96 h) on virus nucleic acids copy number stability and the use of virus infectivity



	Reduction factor
PreAnalytiX	10E+5.72
Norgen	10E+2.90
UTM	10E+2.38
VPM	10E+2.34

Substance	Concentration	Reduction factor
TMA-D	0.03 %	10E+3.53
	0.015 %	10E+2.50
	0.0075 %	10E-0.66
TMA-P	0.1 %	10E+5.72
	0.05 %	10E+2.14
	0.01 %	10E+0.08

Fig. 4. (A) Stabilization of RSV B RNA by swab systems and saliva collection devices at RT and 37 °C during storage of up to 96 h. Data shown refer to two independent experimental series with three replicates each. (B and C) Infectivity of RSV B in the tested swab systems or saliva collection devices (B) or with respective additives (C), by cultivation in A549 cells for 72 h, determined by RT-qPCR. Cell culture medium (Medium) spiked with the same viral load as used for spiking the collection devices served as positive control. Log10 reduction factors were calculated in relation to this positive control. Data shown refer to two independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per system or device.

deactivating additives were examined. The tested conditions represent possible clinically relevant scenarios from collection to analysis. As in the previous study [7], the lowest virus spike-in concentration in this pre-analytical study was set at the applied test detection limit. Each of the five listed respiratory viruses were tested for their viral nucleic acid copy number recovery rate in four commonly used saliva and swab systems. These systems were chosen because of their high capability of stabilizing SARS-CoV-2 RNA [7].

In our study, viral RNA and DNA from samples stored in saliva collection devices were more stable than in transport swab systems, which could be attributed to the stabilizing solution chosen by the manufacturers. Transport swab systems are manufactured for collection, transportation, preservation and long-term storage of nasopharyngeal, oropharyngeal or anterior nares specimen. They are intended to be used for viral molecular diagnostic testing by trained personnel [8]. Viral specimen collected with these transport swab systems can be further cultivated post collection. In contrast, the tested saliva collection devices are non-invasive sample collectors designed for human saliva collection, stabilization, transport and storage but not for virus cultivation [9,10]. However, the Norgen device was not able to stabilize at the lowest virus copy spike-in concentration of adenovirus stored at both applied storage temperatures, RT and 37 °C as well as the other higher concentrations, indicating reduced stabilization capacity for low viral copy numbers.

We were unable to show any differences between the viruses influenza, SARS-CoV-2, RSV and adenovirus – neither in the stabilization up to 4 days storage at RT and 37 °C nor in the virus infectivity inactivation properties of the tested solutions for these viruses. The only exception was the temperature independent stabilization of adenovirus stored in UTM where the measured viral DNA copy number was higher after 96 h than at timepoint zero. This could be due to the nature of the flocked swab which is provided by the company. The non-enveloped adenovirus might be increasingly released from the swab into the medium with increasing storage durations (e.g. after 96 h). This effect of UTM could not be detected using enveloped viruses under the experimental conditions tested in this study.

Few published studies address pre-analytical aspects such as storage conditions during diagnostic workflows for respiratory viruses. Krafft et al. showed the effective and suitable storage of influenza A, B and adenovirus in 100% ethanol at ambient temperature for up to six months [13]. In a second study, Druce et al. detected a loss of viral nucleic acids in influenza specimens stored in the same UTM swab device from Copan after four days of storage at 37 °C. This result is in line with our findings regarding influenza A and B. However, compared to our results they observed no decrease of viral nucleic acid after four days storage of UTM specimen at ambient temperature [14]. We measured a slight decrease for influenza A and an enhanced decrease for influenza B viral copy numbers when stored in UTM at RT up to 96 h. Compared to Druce et al., our RT (23.0 – 24.0 °C) was slightly higher than their ambient temperature (22 °C). This small difference in temperature should not be responsible for the higher instability of the viral RNA observed in our

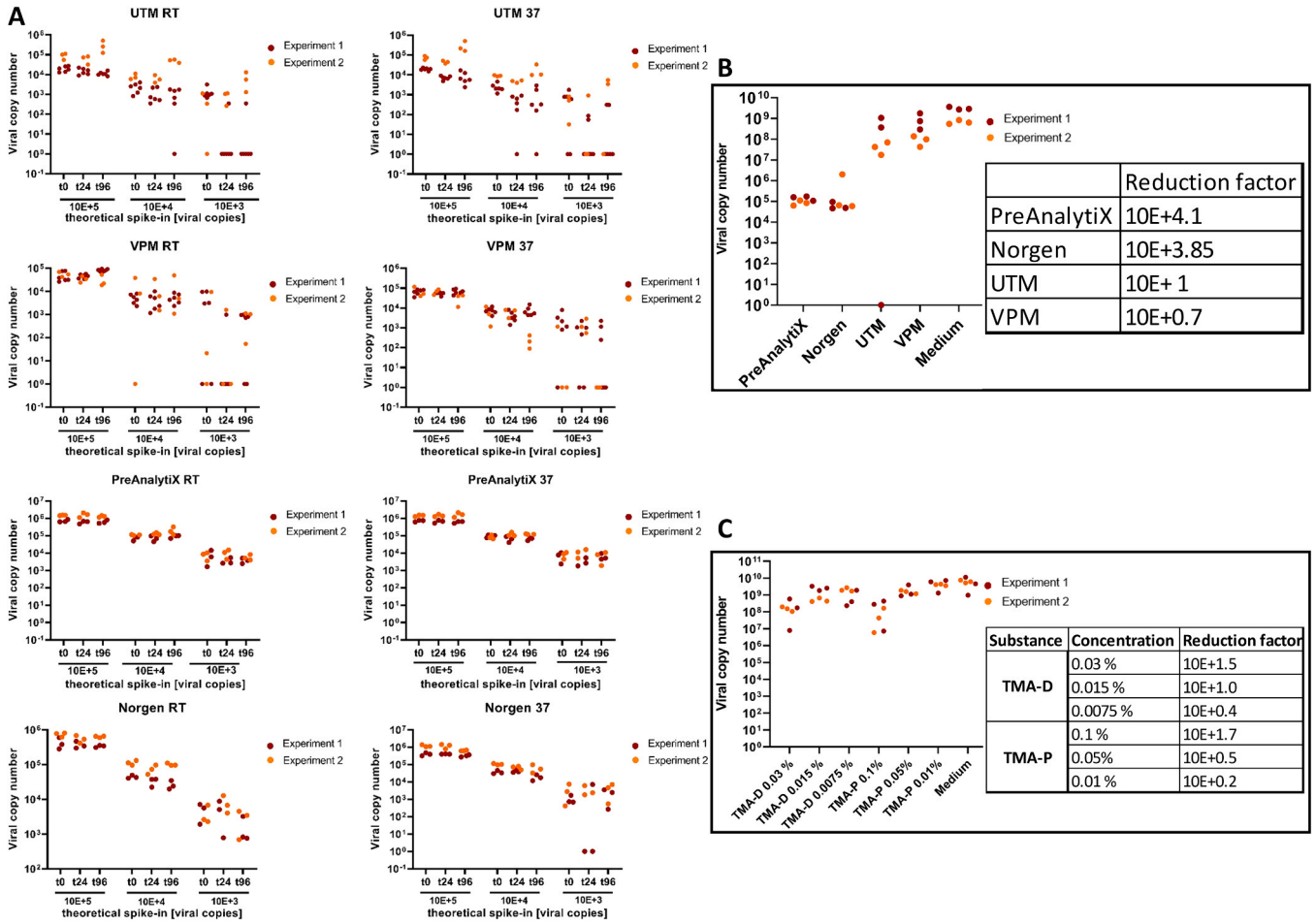


Fig. 5. (A) Stabilization of Adenovirus DNA by swab systems and saliva collection devices at RT and 37 °C during storage for up to 96 h. Data shown refer to two independent experimental series with either six or three replicates. (B and C) Infectivity of Adenovirus in the tested swab systems, saliva collection devices (B) or with respective additives (C) by cultivation in VeroE6 cells after 72 h, determined by RT-qPCR. Cell culture medium (Medium) spiked with the same viral load as used for spiking the collection devices served as positive control. Reduction factors were calculated in relation to this positive control. Data shown refer to two independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per system or device.

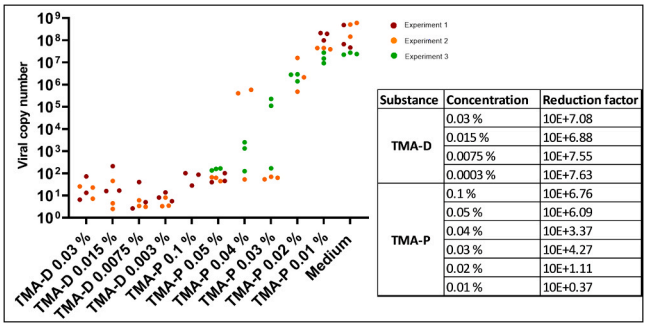


Fig. 6. Infectivity of SARS-CoV-2 treated with two medium additives. Virus infectivity was determined by VeroE6 cell cultures incubated for 72 h, followed by RT-qPCR for virus RNA copy number measurements. Cell culture medium (Medium) spiked with the same viral load as used for spiking medium containing additives served as positive control. Log10 reduction factors were calculated in relation to positive control. Data shown refer to three independent experimental series with three replicates each. Copy numbers were extrapolated to the total amount of viral copies per system or device.

study. Summer et al. reported nucleic acid stability of several respiratory viruses including influenza A, RSV and adenovirus at temperatures up to 28 °C and storage durations up to 28 days in UTM [15].

Falsey and Walsh described the thermolability of RSV detected by a rapid loss of viral particle titer determined by viral infectivty at RT without mentioning the specific medium composition [16]. While we were focusing on molecular testing, we observed a high variability of all RT-qPCR results for both RSV A and B in swab systems UTM and VPM stored for up to 96 h at RT and at 37 °C. In contrast to the UTM and VPM results obtained in our study, the storage conditions used here did not have that huge impact on the stability of RSV A and B when the virus was spiked-in in saliva collection devices PreAnalytiX and Norgen. The storage medium appears to have a greater effect on specimen preservation than the temperature in our study.

The manual of Clinical Microbiology reported a general stability of adenovirus in UTM and VTM for up to a week at 36 °C and several weeks at RT while not specifying the medium composition [4]. We observed stable results for specimen stored in saliva collection devices and the VPM swab system for up to 96 h independent of storage temperature. A decrease in viral DNA load was only observed for the lowest spike-in concentration stored in swab systems indicating that samples with a low virus load might lead to false negative results after several days of storage in this device.

Besides reliable virus detection, the protection of healthcare personnel is a key aspect. Indeed, the efficacy of viral inactivation is of great importance for preventing respiratory virus transmission. Detergents can destroy the lipid layer of e.g. SARS-CoV-2, influenza or RSV envelopes resulting inloss of cell infectivity and loss of viral nucleic acids' protection against nucleases secreted by hosts [17]. Alcohols,

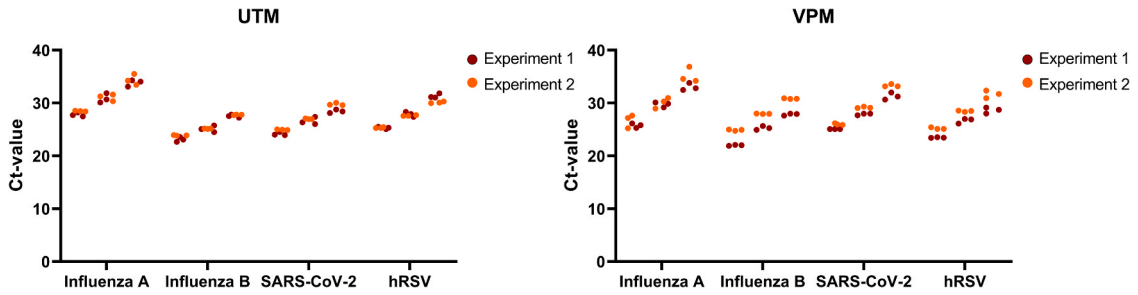


Fig. 7. Multi target RT-qPCR of Influenza A, B, SARS-CoV-2 and RSV A, spiked-in in swab systems UTM and VPM. Samples in triplicates of three different spike-in concentrations of two independent experimental series were collected and analysed via RT-qPCR without further storage directly after spike-in.

such as ethanol, are more effective in inactivating enveloped viruses than non-enveloped viruses [17]. The devices and additives tested in this study differed in their inactivation properties. The non-enveloped adenovirus was less effectively inactivated. The enveloped influenza A H1N1 and influenza Washington B were inactivated by both saliva collection devices tested in this study but were able to replicate after spike-in in UTM and VPM transport swab systems. This finding is not surprising as both swab systems were developed for specimen collection, storage and transport but also for maintaining virus viability and therefore should not interfere with the ability of viruses to replicate in cell culture systems (see manufacturers handbooks). Falsey and Walsh documented the inactivation property of detergents against RSV [16]. While detecting viral nucleic acid copy numbers in our study, the specific viral titer of infectious particles was not measured, only whether they were able to replicate and were thus still infectious. Hambling published that RSV is inactivated after 5 min of storage at 55 °C and only 10% of infective viruses survived after 24 h stored at 37 °C [2]. RSV A could not be completely inactivated by either saliva collection device tested in our studies and also not by the two medium additives. The best inactivation results for RSV B were obtained with the PreAnalytiX device and with the highest concentration of the medium additive TMA-P. A short heat treatment of this thermolabile virus could probably be helpful for additional inactivation when the saliva collection devices are used. This should likely not result in a loss of viral nucleic acids copy numbers and therefore in a loss of information [2]. However, this hypothesis has to be proven in another study.

The complete inhibition of SARS-CoV-2 in vitro infectivity was previously reported when treated with saliva collection devices PreAnalytiX and Norgen [7]. In this study, SARS-CoV-2 could be inactivated completely by the treatment with TMA-D and in a dose dependent manner by TMA-P. Therefore, these additives could be used as a measure to reduce the risk of healthcare personnel for SARS-CoV-2 infections when working with specimen collected in devices which do not sufficiently inactivate virus infectivity, such as the swab systems tested here.

The adenovirus was resistant to inactivation by the tested medium additives similar to the swab systems tested in our study, associated with an almost unhampered replication in cell cultures. According to the definition of the European Agency for the Evaluation of Medicinal Products (EMA), adenovirus was sufficiently inactivated by both saliva collection devices by reducing virus replication by 10E+ 4 [12]. Thus, the use of the PreAnalytiX and Norgen devices should be suitable for inactivation of adenovirus although virus replication in cell cultures could be observed at virus copy number spike-in levels of 10E+ 5. Adenoviruses are highly resistant against chemical and physical treatments [3]. Possible treatments for inactivation are household bleach, heating to 56 °C for 30 min or 60 °C 2 min and autoclaving [4], which however are not compatible with RNA/DNA based diagnostic tests.

Overall, our study provided crucial new insights with respect to respiratory virus nucleic acid copy number stability and infectivity inactivation, which could help the implementation of multi-target virus testing workflows including direct nucleic acid amplification approaches. The detection of the four viruses influenza A and B, SARS-CoV-2 and RSV A, which were spiked simultaneously into the swab systems UTM and VPM without further storage, was reproducible in two independent experimental series. These therefore appear to be suitable for use in healthcare centers by trained personnel. Furthermore, the time required for processing specimens for virus testing can be greatly reduced by avoiding a full RNA isolation and separate reverse transcriptase step.

At this stage, we can summarize that appropriate specimen collection, storage, transport and handling are of great importance in the vulnerable pre-analytical phase to obtain valid diagnostic results. The choice of a suitable saliva and swab systems or another suited collection device depends on the downstream application and respective assays used. Specifying, developing, verifying and validating pre-analytical workflows should therefore be an essential part of analytical virus test development. Using multi-target detection assays may lay the foundation for future implementations of testing strategies. Finally, inactivating additives like TMA-D or TMA-P can reduce the risk of infection for healthcare personnel. Further viruses of high relevance should be tested in this context of multi-target detection assays.

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CRediT authorship contribution statement

MH: performed experiments, analysed and interpreted data, contributed to study design, writing and proof reading of the manuscript. FK: interpreted data, contributed to study design, writing and proof reading of the manuscript. TV: interpreted data, contributed to study design, writing and proof reading of the manuscript. UO: contributed to study design, proof reading of the manuscript KZ: contributed to study design, proof reading of the manuscript. All authors have approved the final article.

Declaration of Competing Interest

Franziska Kaiser, Uwe Oelmüller, Thorsten Voss, Katrin Rodenkirchen are employees of QIAGEN GmbH Hilden, Germany. Uwe Oelmüller and Thorsten Voss participate in QIAGEN's regular long term incentive program (LTIs). Uwe Oelmüller is Co-chair of PreAnalytiX management board. Kurt Zatloukal is co-founder and CEO of Zatloukal Innovations GmbH.

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