National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

RIVM
Centre for Infectious Diseases
A. van Leeuwenhoeklaan 9
3721 MA Bilthoven
Postbus 1
3720 BA Bilthoven
www.rivm.nl

5.1.2e
info@rivm.nl

Evaluation of different saliva collection systems for molecular detection of SARS-CoV-2

Evaluation Report
Final

Authors:
5.1.2e
5.1.2e
5.1.2e
5.1.2e

Version history
2 – 10/07/2020: system 13 added

Introduction

Most frequently used specimens for SARS-CoV-2 detection are nasopharyngeal (NP) and oropharyngeal (OP) swabs, with NP in general more frequently and for a longer period positive than OP. Because of shortages of NP swabs, alternatives are sought. In The Netherlands for many years in the surveillance of mumps, measles and rubella and pneumococci oral fluid is being used for molecular detection of the virus or bacterium. Saliva can be used as alternative convenient specimen for SARS-CoV-2 diagnostics as has been shown in the literature and by our own investigation in the 'children study'. In the later study we used Oracol S10 sponge transported on ice and immediate freezing at -80°C after processing at the laboratory, which requires dedicated handling in a biohazard cabinet and centrifugation. A number of laboratories have responded that this is especially problematic for high throughput analysis There are various alternative saliva collection systems that potentially can be used for molecular detection of SARS-CoV-2. Some of them include nucleic acid preservatives that can be helpful when saliva is transported at ambient temperature e.g. overnight. The various systems are evaluated if the sensitivity is higher, similar or lower compared to the gold standard method as described above that is being used now.

Material & Methods

Saliva has been collected from a SARS-CoV-2 negative donor by using a saliva collector Oracol S10 (Malmed, Worchester, United Kingdom). For spiking we used inactivated SARS-CoV-2 (hCoV-19/Netherlands/Noord_Braband_0117R/2020) in MEM with Hanks' salts; heat inactivated at 60°C for 2 hours; 5.62*10^7 TCID50/ml or 8.26*10^8 digital copies RdRP positive strand RNA/ml. All the information of the saliva collection systems is described in table 1 and an overview of the systems in figure 1. The final concentration SARS-CoV-2 used in saliva and viral transport medium GLY is 5.62*10^2 TCID50/ml or 8.26*10^3 digital copies RdRP positive strand RNA/ml. To each saliva collection system 1 ml spiked saliva (n=3) or spiked viral transport medium GLY (n=3) are added and processed as described by the manufacturer. After incubating 2, 8, 24 and 48 hours at room temperature and at 4°C, from each specimen a sample of 200 µl was drawn and mixed with 275 µl MagNA Pure lysis buffer with EAV included and 450 µl was extracted on a MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) and eluted in a volume of 50 µl. The E-gene/EAV multiplex PCR was used to test inhibition of EAV amplification. E-gene and RdRp-gene primers and probes were as described by Corman et al (1). EAV primers and probe were as described by Scheltinga et al (2).Reaction condition are described in Table 2 and 3. The Equine Arteritis Virus (EAV) is used as standard internal control for the qRT-PCR to control for inhibition.

***Table 1**. overview of saliva collection systems; supplier, manufacture and model*

System	Supplier	Manufacture	Model
1	Malmed	Malmed, UK	Oracol S10 (no preservation buffer)
2	Isogen	INVITEK Molecular, Germany	SalivaGene Collector (with dried preservation buffer SLK in collection tube)
3	Sanbio	NORGEN BIOTEK Corp, Canada	Saliva RNA Collection and Preservation Device (preservation buffer not used)
4	Sarstedt	Sarstedt, Germany	Salivette® REF 51.1534 (With saliva absorbing pad, no preservation buffer)
5	Westburg	Isohelix, UK	Custom order (Collection tube and funnel without preservation buffer)
6	Greiner	Greiner	Greiner Bio-One Saliva Collection System (separate dry preservation reagent in vacuum tube used and saliva flow stimulant buffer not used)
7	BioConnect	Salimetrix, State College, USA	Saliva Collection Ad (no preservation buffer)
8	Malmed	Malmed, UK	Oracol S14 (no preservation buffer)
9	Sanbio	NORGEN BIOTEK Corp, Canada	Saliva RNA Collection and Preservation Device (with preservation buffer in separate twist-off ampoule)
10	Greiner	Greiner	Greiner Bio-One Saliva Collection System (separate dry preservation reagent in vacuum tube and saliva flow stimulant buffer used)
11	Westburg	Isohelix, UK	GeneFiX DNA saliva collection (with preservation buffer already in collection tube)
12	Westburg	Isohelix, UK	GeneFiX RNA saliva collection (with preservation buffer already in collection tube)
13	ProDiag B.V.	Xiamen Zeesan Biotech Co., Ltd., China	Saliva RNA Sample Collection Kit, Custom order (Collection tube and funnel without preservation buffer

Figure 1. *The various collection systems used in the evaluation.*

Table 2*. For the SARS-CoV-2 the primers and probes obtained from Biolegio were premixed at a final concentration of 10 µM primers and 5 µM probes.*

E-gene/EAV qRT PCR	**µl**	**RdRp**-gene qRT-PCR	**µl**
4x Taqman Fast Virus MM	5	**4x Taqman Fast Virus MM**	5
E+EAV Mix	3	**RdRp Mix**	3
PCR grade water	7	**PCR grade water**	7
Specimen nucleic acid	5	**Specimen nucleic acid**	5
Total volume	20	**Total volume**	20

Table 3*. Amplification temperature protocol LC480 mark II.*

PCR Program	**Segment** number	**Temp Target (°C)**	**Hold** Time **(sec.)**	**Slope (°C/sec.)**	**Acquisition mode**	
Reverse Transcription	**1**	**50**	**900**	**EXTERNAL**		
Denaturation/Inactivation	**1**	**95**	**120**	**EXTERNAL**		
Denaturation	1	95	60	4.4	None	LC 480
Amplification	1	95	10	4.4	None	
(cycles:50)	2	**60**	30	2.2	Single	
Cooling	1	40	30	4.4	None	

To be accepted, the saliva collection systems should fulfil the following criteria compared with Malmed saliva collector Oracol S10 (System 1):

1. No or minimal release of qRT-PCR inhibitors
2. Similar or better performance in collecting and releasing SARS-CoV-2
3. Similar or better ease of handling by those who collect the specimens and lab workers

Results

Presence of inhibition factors in the different saliva collection systems

The spiked saliva and spiked viral transport medium GLY are tested in EAV qRT-PCR to control for inhibiting factors released from the material or due to the liquid/buffer provided by the manufacturer (figure 2). The RNA extracted from these samples was tested in a duplex E-gene and EAV qRT-PCR . The results of the E-gene qRT-PCR were used for analyzing the influence of the various collection systems on the sensitivity of detection of SARS-CoV-2 in saliva collected in the various collection systems compared with gold standard collection system 1 (Figure 3).

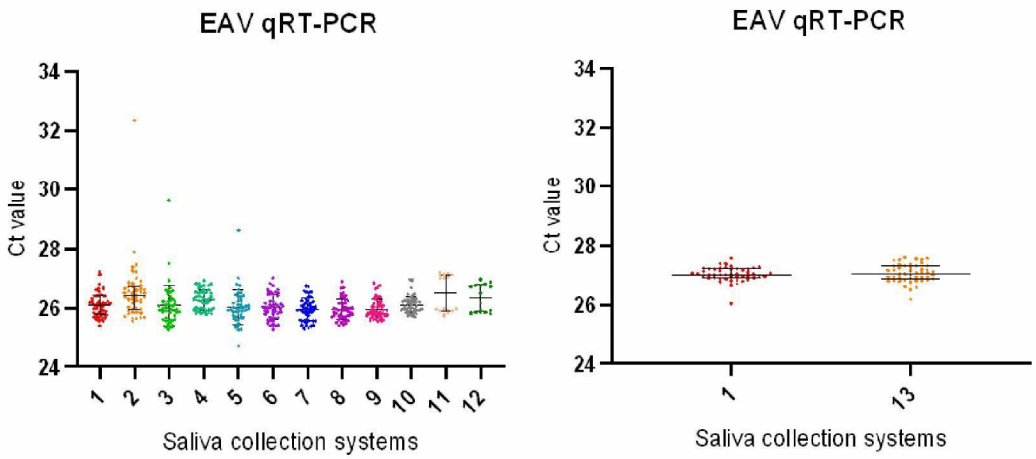

Figure 2. *EAV qRT-PCR results to determine if there are inhibiting factors present in the different saliva collection systems compared with the saliva collection system 1. Data points are for room temperature, 4°C and all time points of collection aggregated. System 1 was tested separately again with system 13 that was received at a later stage.*

In general there is no inhibition in all the saliva collection systems compared with saliva collection system 1. In saliva collection systems 2, 3 and 5 there is one sample that is inhibited. In saliva collection systems 11 and 12 there are 2 groups of results. de top half of the results is saliva with higher Ct values compared to the bottom half which is viral transport medium. Storage temperature and length of storage did not affect amplification of EAV in the added lysis buffer.

The different saliva collection systems have an effect on SARS-CoV-2 detection

The 2, 8, 24, 48 hours incubated saliva and viral transport medium GLY at room temperature and at 4°C are tested in the SARS-CoV-2 qRT-PCR. This will show whether any of the different saliva collection systems, storage temperatures and incubation times have an effect on SARS-CoV-2 in saliva or viral transport medium for comparison. These samples are tested for E-gene and RdRp-gene.

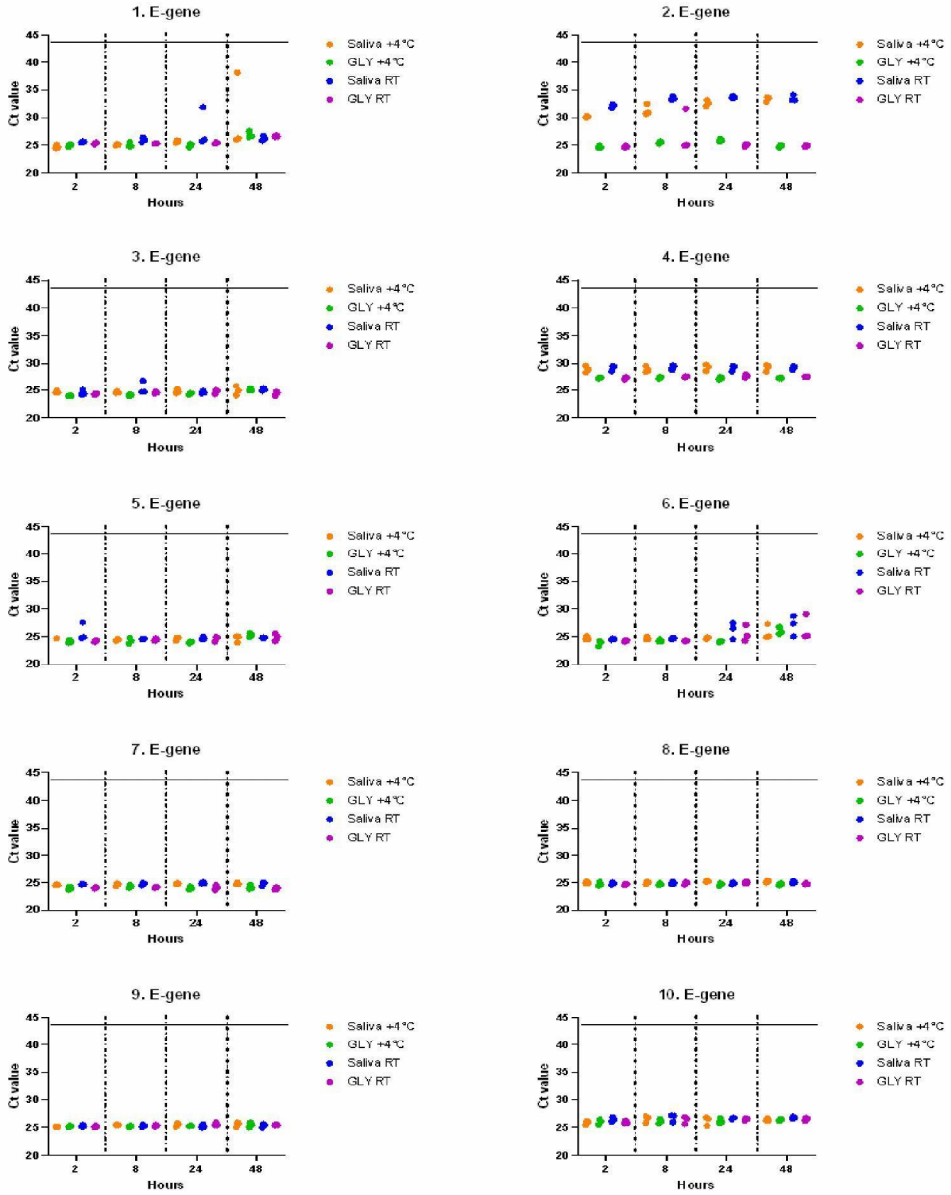

Figure 3. *E-gene qRT-PCR results of saliva and viral transport medium GLY incubated in the saliva collection systems 1 to 10 for 2, 8, 24 and 48 hours at room temperature and 4°C.*

The results of the E-gene qRT-PCR (figure 3) show that saliva collection systems 3, 5, 7, 8, 9 and 10 have similar results compared with system 1. De saliva samples in system 2 have a Ct-value that is ±5 higher compared with system 1. The combination dried preservation buffer and saliva show degradation of SARS-CoV-2 compared with the combination of dried preservation buffer and viral transport medium GLY. In System 4 saliva as well

viral transport medium GLY has a Ct-value that is 3-4 higher compared with system 1. In system 5, saliva 4°C, 2 hours incubation is only tested in one fold. In system 6 after incubating for at least 24 hours the saliva as well viral transport medium GLY show degradation of SARS-CoV-2 RNA at room temperature as indicated by increasing Ct values over time.

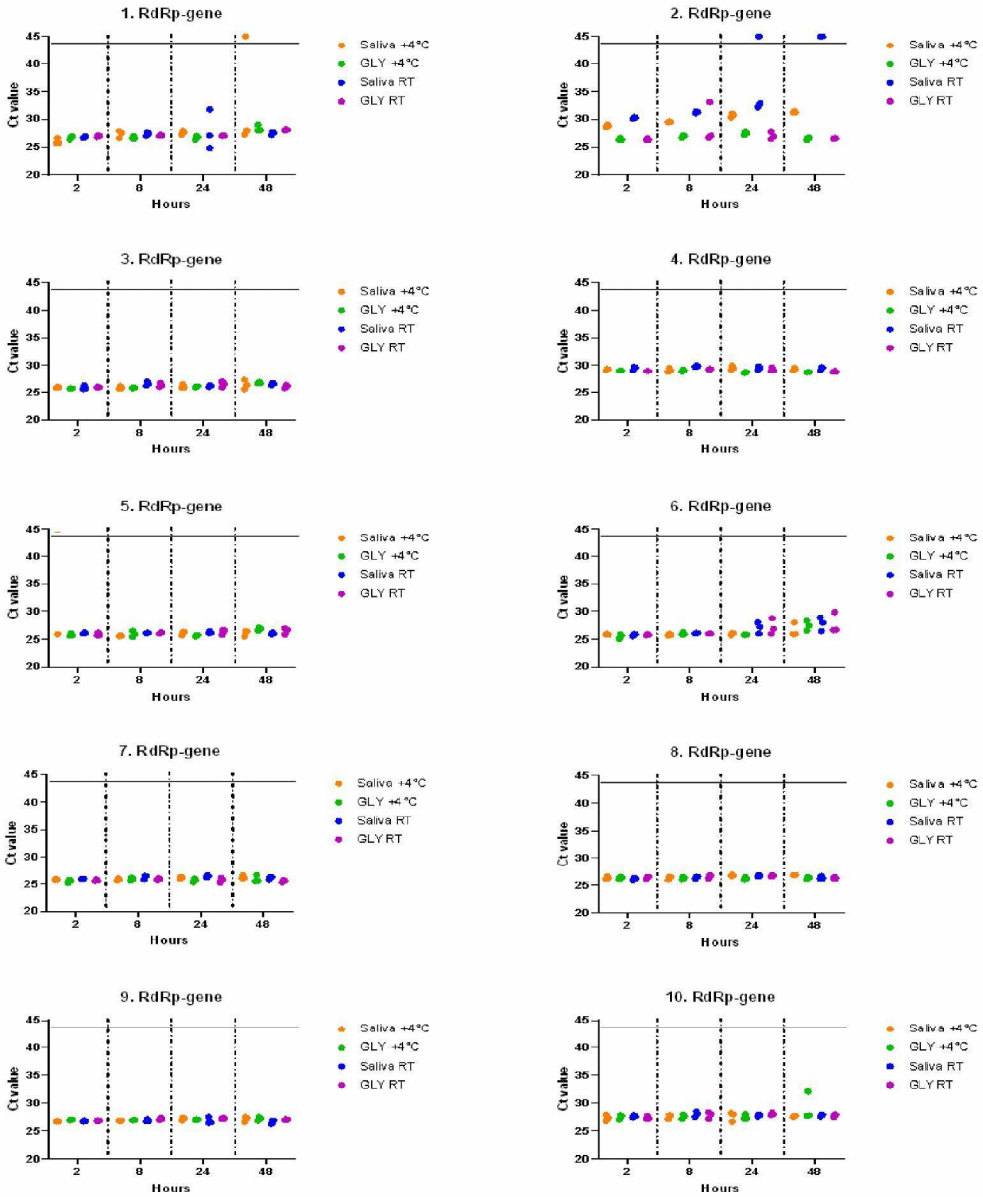

Figure 4. *RdRp-gene qRT-PCR results of saliva and viral transport medium GLY incubated in the saliva collection systems 1 to 10 for 2, 8, 24 and 48 hours at room temperature and 4°C.*

The results of the RdRp-gene qRT-PCR (figure 4) show that saliva collection systems 3, 5, 7, 8, 9 and 10 have similar results compared with system 1. De saliva samples in system 2 has a Ct-value that is ±5 higher compared with system 1. The viral transport medium GLY has similar results. in System 4 saliva as well viral transport medium GLY has a Ct-value that is 3-4 higher compared with system 1. In system 5, saliva 4°C, 2

hours incubation is only tested in one fold. In system 6 after incubating for at least 24 hours the saliva as well viral transport medium GLY there is show degradation of SARS-CoV-2 RNA at room temperature as indicated by increasing Ct values over time. The results for E-gene and RdRP-gene detection are very similar

Saliva collection systems 11 and 12 were tested separate from the other saliva collection systems (figure 5). Saliva collection system 1 was tested next to systems 11 and 12 as reference.

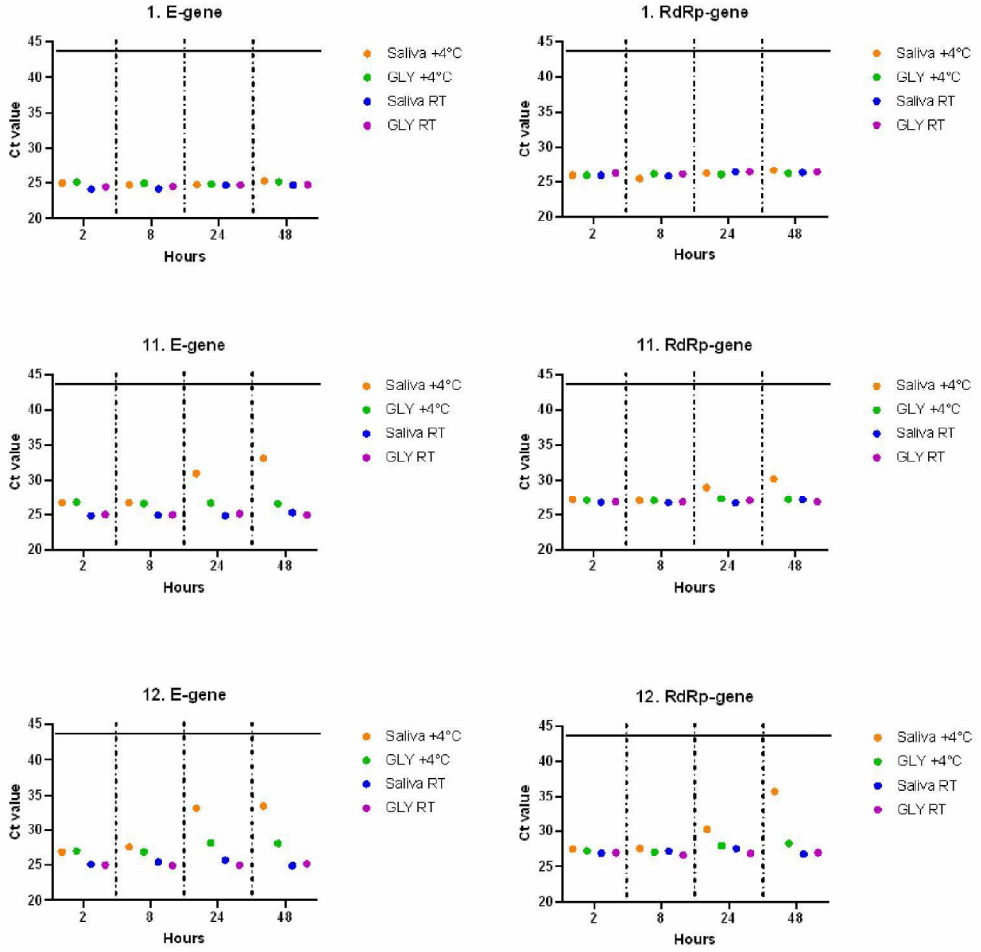

Figure 5. *E-gene and RdRp-gene qRT-PCR results of saliva and viral transport medium GLY incubated in the saliva collection systems 11 and 12 for 2, 8, 24 and 48 hours at room temperature and 4°C.*

The results of the E-gene and RdRp-gene qRT-PCR for saliva collection systems 11 and 12 stored at room temperature, as recommended by the manufacturer, are largely similar compared with 'gold standard' system 1. Because laboratories store most specimens routinely at 4°C, we tested the effect of storage at 4°C despite the fact that the manufacturer does not recommend this. Indeed, the results of the E-gene qRT-PCR for saliva collection systems 11 and 12 are not similar compared with system 1. The results show that in system 11 and 12 after 2 hours at 4°C for saliva as well viral transport medium GLY in buffer a Ct-value is observed that is 1-2 higher compared with system 1 in the E-gene qRT-PCR. After 24 hours at 4°C the saliva samples generate a Ct-value that is more than 5 higher compared with system 1. The results for the RdRp-gene show that after 8 hours of incubation at 4°C the SARS-CoV-2 RNA in saliva is adversely affected in both system 11 and 12.

Saliva collection system 13 is tested separate from the other saliva collection systems (figure 6) because it was received at a later stage as a possible replacement for system 5. Saliva collection system 1 was tested next to systems 13 as reference.

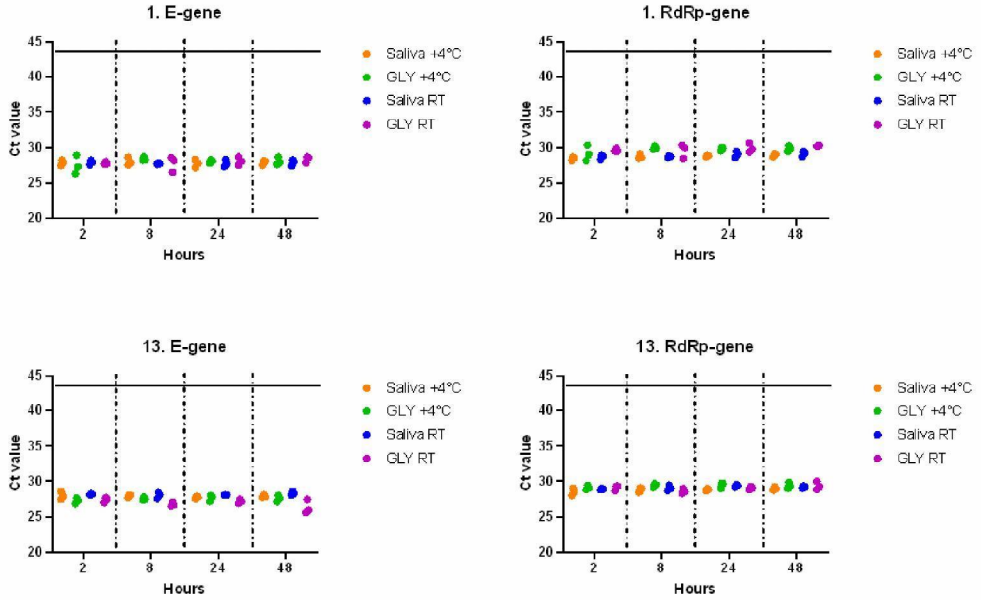

Figure 6. *E-gene and RdRp-gene qRT-PCR results of saliva and viral transport medium GLY incubated in the saliva collection system 1 and 13 for 2, 8, 24 and 48 hours at room temperature and 4°C.*

The results of the E-gene and RdRp-gene qRT-PCR show that saliva collection systems 13 have similar results compared with system 1.

As a control, SARS-CoV-2 spiked saliva (n=3) and spiked viral transport medium GLY (n=3) is tested in the E-gene and RdRp-gene qRT-PCR before and after freezing the samples (Figure 7).

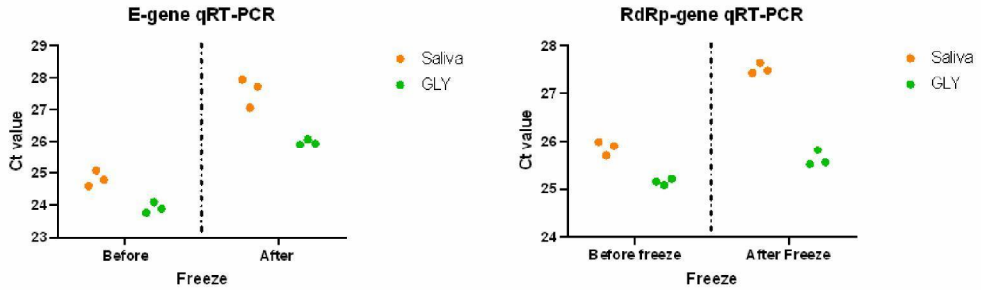

Figure 7. *Saliva results of the E-gene and RdRp-gene qRT-PCR before and after freezing the samples, compared with viral transport medium GLY.*

This is done to show whether RNA reduction is caused by freezing the saliva in comparison with viral transport medium GLY. The results of the E-gene qRT-PCR after freezing the samples show that RNA detection is reduced in saliva and viral transport medium as indicated by an increase of ±2 Ct values. In the RdRp-gene qRT-PCR the reduction of RNA in saliva is 1-2 Ct-Value and in viral transport medium GLY <1 Ct-Value. Overall, Ct values with Saliva are higher than with GLY, possibly indicating some degradation of RNA in saliva. Nevertheless, the results show that freeze thawing of saliva should be avoided if possible.

Ease of use in the field as well in the laboratory

In the laboratory various aspect were examined, see table 4.

Table 4. *The answers of the various aspects that were examined.*

Saliva system	Aspects								
	1[1]	2[2]	3[3]	4[4]	5[5]	6[6]	7[7]	8[8]	9[9]
1	Yes	No	No	No	No	Yes[10]	Yes	Yes	Yes
2	No	No	Yes	No	Yes[11]	Yes	No	Yes	No[12]
3	No	No	Yes	Yes	Yes[11]	Yes	No	Yes	No[12]
4	No	No	No	No	No	No	Yes	Yes	No[12]
5	No	No	No	No	No	Yes	No	Yes	Yes
6	No	Yes	No	Yes	No	Yes	No	Yes	No[12]
7	No	No	No	No	No	Yes	No	No	Yes
8	Yes	No	No	No	No	Yes[10]	Yes	No	Yes
9	No	No	Yes	Yes	Yes[11]	Yes	No	Yes	No[12]
10	No	Yes	No	Yes	No	Yes	No	Yes	No[12]
11	No	No	Yes	No	No	Yes	No	Yes	No[12]
12	No	No	Yes	No	No	Yes	No	Yes	No[12]
13	No	No	No	No	No	Yes	No	Yes	Yes

[1] Practical for baby's, toddlers and preschoolers
[2] Have included stimulants for salivation
[3] Have included agents that protect RNA/DNA
[4] Additional action required for the sample collection team
[5] Safety issues for collection team
[6] Can safely be worked with in the laboratory
[7] Centrifuging needed for saliva collection from the device
[8] Pipetting needed for aliquoting
[9] The system is suitable for both molecular and antibody detection
[10] With proper instructions
[11] Tipping over and spill hazard
[12] Has to be tested if it's suitable for molecular and antibody detection

The results show that saliva collection systems 1 and 8 are practical for baby, toddlers and preschoolers. Systems 6 and 10 have simulants for salivation. This is not practical for baby, toddlers and preschoolers because it is in liquid form. Systems 2, 3, 9, 11 and 12 include preservation agents to protect the DNA/RNA from degradation. In systems 2, 9, 11 and 12 the results of the E-gene and RdRp-gene qRT-PCR show that their preservation agents have no added value for SARS-CoV-2 detection.
The additional action required for the sample collection team with systems 3 and 9 is transferring the preservation buffer into the tube where the saliva is collected in. System 9 uses a rather soft plastic ampoule from which the top has to be twisted off to be opened. During this handling the liquid leaks over your fingers. A larger ampoule that is not filled to the top would be of benefit. Systems 2, 3 and 9 have (also) another safety issue for the sample collection team. The safety issue is that is has tipping over and spill hazard because these systems do not have a leakage prevention like system 5, 11, 12 and 13 has. System 4 has a safety issue for the lab workers. After centrifuging this system the tube with saliva absorbing material has to be removed; this tube is possible contaminated with SARS-CoV-2. Although system 8 is an improvement compared to the original system 1 because system 8 can be put in the centrifuge without prior handling in the lab, too much saliva in the sponge causes overflow/spill when the collection tube is removed from the system. Systems 1, 5, 7, 8 and 13 are suitable for molecular and antibody determination. All the other saliva collection systems need to be tested properly for antibody determination.

Additional information on infectious virus and RNA of SARS-CoV-2 in saliva compared with viral transport medium GLY

For additional experiments saliva was collected from a voluntary donor by using a saliva collector S10 (Malmed, Worchester, United Kingdom). For spiking we used infectious SARS-CoV-2 (hCoV-

19/Netherlands/Noord_Braband_0117R/2020); 5.62*10^7 TCID50/ml or 8.26*10^8 digital copies RdRP positive strand RNA/ml. The final concentration SARS-CoV-2 used in saliva and viral transport medium GLY is 5.62*10^6 TCID50/ml or 8.26*10^7 digital copies RdRP positive strand RNA/ml The spiked saliva (n=3) and viral transport medium (n=3) is transferred into 15 ml Greiner tubes (Greiner Bio-one). After incubating 2, 8, 24, 48, 144 and 216 hours at room temperature and at 4°C, from each sample 200 µl was drawn and mixed with 275 µl lysis buffer with EAV included and 450 µl was extracted on a MagNA Pure 96 Instrument (Roche) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche) and eluted in a volume of 50 µl. The E-gene/EAV multiplex PCR was used to test inhibition of EAV amplification. E-gene and RdRp-gene primers and probes were as described by Corman et al (1). Reaction condition are described in Table 2 and 3. To determine the TCID50/ml Log reduction, 25 µl from each sample was used for infectious virus titration. The TCID50 determination is performed on Vero-E6 cells (ATCC CRL-1586) in duplicate. The virus titration is incubated for 4 days at 35°C with 5% CO_2.

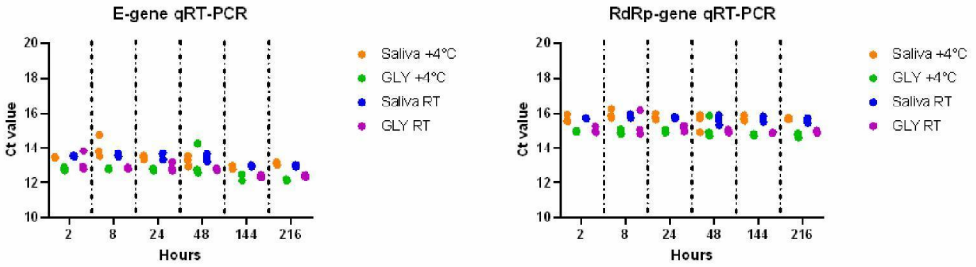

Figure 8. *E-gene and RdRp-gene qRT-PCR results of saliva and viral transport medium GLY incubated for 2, 8, 24, 48, 144 and 216 hours at room temperature and 4°C.*

The E-gene and RdRp-gene qRT-PCR results show that after 216 hours at room temperature or 4°C there is no reduction of SARS-CoV-2 RNA in saliva and viral transport medium GLY.

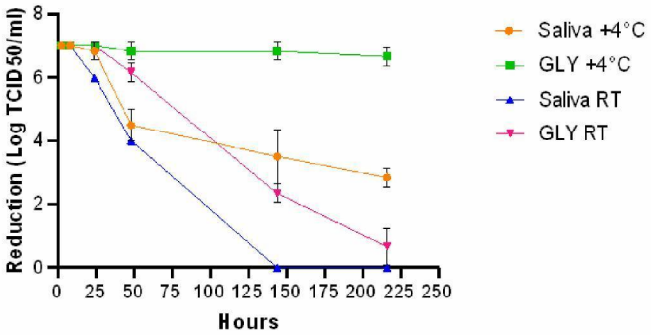

Figure 9. *Log TCID50/ml reduction of SARS-CoV-2 virus in saliva and viral transport medium at room temperature and 4°C.*

The results of the virus titration show that virus in viral transport medium GLY at 4°C has no Log10 reduction after 216 hours incubation. After 48 hours at room temperature the virus in viral transport medium GLY has a 0.5 Log10 reduction, after 144 hours 4.5 Log10 reduction and after 216 hours 6 Log10 reduction. Saliva at 4°C shows after 48 hours 2.5 Log10 reduction, after 144 hours 3.5 Log10 reduction and after 216 hours 4 Log10 reduction. After 24 hours at room temperature, saliva has a 1 Log10 reduction, after 48 hours; 3 $Log10^3$ reduction and after 144 hours; 7 Log10 reduction. These results show that infectious SARS-CoV-2 rapidly reduces in saliva but that RNA remains detectable at the same level up to 216 hours when stored at room temperature or 4°C.

Conclusion

All the saliva collection systems (1 to 13) show no inhibition in the EAV qRT-PCR. With saliva collection systems 2, 3 and 5 there was 1 sample inhibited; the cause of inhibition of these samples is unknown. Systems 3, 5, 7, 8, 9, 10 and 13 showed similar SARS-CoV-2 detection results compared with saliva collection system 1. With the other systems (2, 4 and 6; 11 and 12 at 4°C only) SARS-CoV-2 detection was adversely affected, resulting in higher Ct values compared to system 1. Therefore these systems are not recommended. Because of reduced ease of use, systems 7 and 10 are not recommended to be used for baby's, toddlers and preschoolers. Systems 3 and 9 have tipping over and spill hazard and therefore not recommended. Therefore, systems 3, 7, 9 and 10 are excluded. Although system 8 is easy to use at specimen collection and in the laboratory, the overflow hazard makes this system less suitable for wider use. Although systems 11 and 12 could be used stored at room temperature, the requirement not to store at 4°C makes it prone to error in the hectic SARS-CoV-2 testing environment in the lab. Furthermore, addition of preservation buffer has no added value. Therefore, the systems that are recommended after this evaluation are systems 1 and 5 or 13; eventually to be used in combination with each other or using the sponge assisted version of system 5 and 13. Storage temperature can be either room temperature or 4°C, but rapid delivery at the laboratory after specimen collection is recommended.

References

1. Corman VM, Landt O, Kaiser M, Molenkamp R, 5.1.2e, Chu DK, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DG, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, 5.1.2e, Reusken C, Koopmans MP, Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill. 2020 Jan;25(3):2000045.
2. S.A. Scheltinga, K.E. Templeton, M.F.C. Beersma, E.C.J. Claas Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. Journal of Clinical Virology 33 (2005), 2004 Aug; 17: 306-311.