Development of In-house Viral Nucleic Acid Extraction Media for COVID-19 Testing by RT-qPCR

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Abstract

Quantitative Reverse Transcription PCR (RT-qPCR) tests are widely used for COVID-19 diagnosis. Samples obtained from COVID-19 patients must be transported and preserved until PCR tests are performed. Various viral transport media (VTM) have been used to transfer and preserve Nasopharyngeal swab samples collected from patients for PCR tests during the COVID-19 pandemic. Some are just for the preservation of viral material during transport to the test center, some contain agents for quick extraction of viral nucleic acids. However, there is not enough and clear data regarding recipes of media used for nucleic acid extraction. In this study, we produced different extraction media by combining various reagents including Tris-Cl, Tris-EDTA, Triton-X and guanidinium thiocyanate (GITC). A total of 20 different media combinations were prepared and tested. First, we tested the RT-qPCR amplification profile of extraction media using RNA obtained from COVID-19 patients to observe if these interfere with PCR. We observed that GITC at 4M inhibits PCR amplification. It was also demonstrated that Triton-X in extraction media does not interfere with RT-qPCR amplification. Next, extraction media were tested in COVID-19 RT-qPCR tests using swabs obtained from 5 COVID-19 patients, directly immersed into extraction media. It was shown that Triton-X in may be used for transportation and preservation of COVID-19 patient samples for RT-qPCR test. At the same time, stability tests were performed to investigate sample stability in these media. Patient samples could be stored at 4 ºC for 24 hours without interfering with RT-qPCR results of COVID-19 patients. In conclusion, multiple alterN/Atives for nucleic acid extraction media that can be prepared in-house were tested and shown to efficiently extract and preserve COVID-19 patients' samples for RT-qPCR testing.

Keywords: COVID-19, Viral Transport Medium (VTM), Nucleic Acid Extraction, RT-qPCR Test

Introduction

Testing for viral diseases begins with the isolation of viral nucleic acid from the virus and then continues with qPCR detection of the disease (Grant et al. 2020). With the COVID-19 pandemic, there has been a significant increase in the number of tests for viral diseases, and a reliable nucleic acid isolation has become essential especially for accurate results in qPCR testing. However, the sample taken from the patient should not be affected by ambient conditions and should give accurate results. Therefore, various VTM (viral transfer medium) and VNAT (viral nucleic acid transformation) formulations have been produced. Since March 2020, qPCR tests demands have increased and in direct proportion with this increase, a shortage of nasopharyngeal swaps, VTM and VNATs occured causing significant bottlenecks in supplies (Smith et al. 2020). In this study, different VNAT combinations were prepared, the most effective formulation was determined comparing the efficiency of the formulations in qPCR studies using patient samples or SARS-CoV-2 RNA from patient samples

Methods & Materials

VNAT Formulations

Table 1. Initial VNAT formulations

	VNAT1	VNAT2	VNAT3
pH	8.32	9.1	7.4
GITC	4 M	5 M	0
EDTA	25 mM	22 mM	0
TritonX-100	3%	1.20%	0.25%
NaCl	0	0	150 mM
Tris-Cl	55 mM	50 mM	10 mM

Methods & Materials

Since most of the VNAT studies given in the literature are not comparable to qPCR studies, initially, three differently formulated VNATs were produced based on the literature (Table 1, Gonzalez-Perez et al., 2010, Kirkland, and Frost 2020, Scallan et al., 2020). After the prepared VNAT contents were autoclaved, they were distributed into tubes in a sterile environment and kept in the dark until used. Ethical approval for use of patient samples was obtained from Ethics Committee for Non-Interventional Studies of Hitit University.

VNAT Formulation Combinations and qPCR Testing

The efficacy of initial VNAT formulations was compared by performing qPCR experiments using the Senteligo SARS-CoV-2 detection kit, one of the tests produced by Eryiğit Companies, and patient samples taken from Hitit University. qPCR reaction mix contains 15 µl of ready-to-use master mix and 5 µl of vNAT solution containing patient sample. qPCR was performed with a hold cycle of 45 °C for 10 minutes, then with a hold cycle of 95 °C for 10 minutes and followed by 35 cycles of 95 °C for 5 seconds and 60 °C for 45 seconds. As a result of initial data, 18 different combinations containing Tris-Cl, Tris-EDTA, Triton-X and guanidinium thiocyanate were formed (Table 2). The efficiencies of these 18 different combinations were compared by setting up qPCR reactions under the same conditions using RNA from COVID-19 patient samples.

Table 2. List of 18 different formulations adjusted separately with Tris-EDTA and Tris-Cl buffer

	Tris-EDTA buffer +		Tris-Cl buffer +
1	3% Triton-X-100	**10**	3% Triton-X-100
2	3% Triton-X-100 + 0,1 M GITC	**11**	3% Triton-X-100 + 0,1 M GITC
3	3% Triton-X-100 + 0,5 M GITC	**12**	3% Triton-X-100 + 0,5 M GITC
4	1% Triton-X-100	**13**	1% Triton-X-100
5	1% Triton-X-100 + 0,1 M GITC	**14**	1% Triton-X-100 + 0,1 M GITC
6	1% Triton-X-100 + 0,5 M GITC	**15**	1% Triton-X-100 + 0,5 M GITC
7	0.5% Triton-X-100	**16**	0.5% Triton-X-100
8	0.5% Triton-X-100 + 0,1 M GITC	**17**	0.5% Triton-X-100 + 0,1 M GITC
9	0.5% Triton-X-100 + 0,5 M GITC	**18**	0.5% Triton-X-100 + 0,5 M GITC

Results

The working principle of the Senteligo SARS-CoV-2 detection kit, which is produced by Eryiğit Companies and is commercially available, basically depends on 3 fluorophores. Of these fluorophores, FAM and HEX give results on the diagnosis of the disease, while CY5 is used as a positive control and indicates proper patient sample collection. qPCR reactions were set up in at least triplicates.

Table 3. Ct results of 18 VNAT combinations

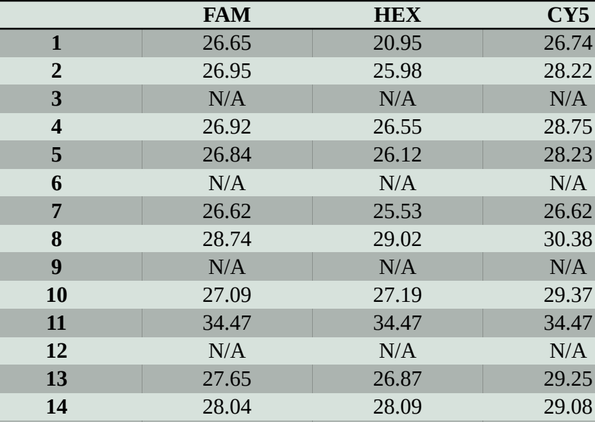
	FAM	HEX	CY5
1	26.65	20.95	26.74
2	26.95	25.98	28.22
3	N/A	N/A	N/A
4	26.92	26.55	28.75
5	26.84	26.12	28.23
6	N/A	N/A	N/A
7	26.62	25.53	26.62
8	28.74	29.02	30.38
9	N/A	N/A	N/A
10	27.09	27.19	29.37
11	34.47	34.47	34.47
12	N/A	N/A	N/A
13	27.65	26.87	29.25
14	28.04	28.09	29.08
15	N/A	N/A	N/A
16	27.86	27.01	28.88
17	30.05	30.05	30.05
18	N/A	N/A	N/A

At first, qPCR experiments were performed using 3 initial VNAT formulations in Table 1. It was found that GITC inhibited PCR amplifications. Based on these data, 18 combinations were prepared and tested. The comparisons of 18 VNAT combinations were made by isolating RNA from samples obtained from patients and creating a pool from these RNAs in order to measure qPCR effectiveness. Unsampled VNAT was used as a control. Ct results obtained from qPCR experiments using 18 VNAT combinations and RNA pool were given in Table 3.

Table 4. Ct results of VNAT formulation #16 using patient samples

	FAM	HEX	CY5
Patient 1	27	29	32
Patient 2	25	28	29
Patient 3	20	26	33
Patient 4	26	30	33
Patient 5	30	NA	33

Based on the qPCR data obtained by using 18 VNAT formulations using pooled RNA, one formulation (#16) was selected for further analysis and tested using 5 patient samples. Ct values obtained by using VNAT formulation #16 using patient samples were given in Table 4. In addition, 2 patient samples were stored at 4 °C for 24 hours, and similar results were obtained after soring at 4 °C.



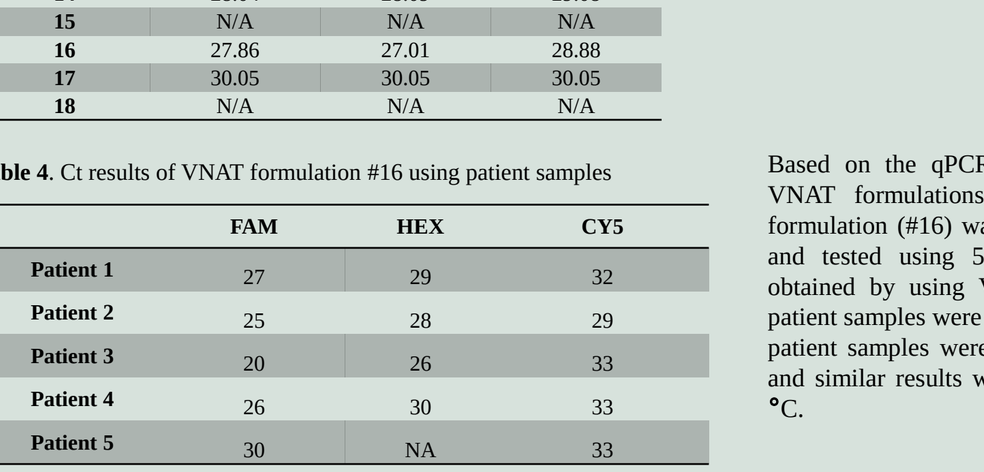


Figure 1. A n example of the qPCR results of VNAT trials with the Bio Rad CFX96 Real Time System C1000 Thermal Cycler

Conclusions

More than a total of 20 VNAT formulations were tested by qPCR by making use of COVID-19 patient samples and it has been observed that several of them can be utilized in qPCR reactions as VNAT solutions. GITC was observed to inhibit PCR amplification.
Among the 18 different combinations of VNAT formulations, the best one was selected, modified and commercially produced by changing the formulation. The final formulation was not given as it was considerred as a trade secret because of the know-how policy of the company.

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