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Evaluation of NaGene COVID-2019 direct PCR kit for SARS-CoV-2 detection

Final Report

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Main findings

- The NaGene COVID2019 direct-PCR kit detects SARS-CoV-2 positive clinical specimens collected in GLY transport medium with a Ct value below approx. 31 in our in-house reference qRT-PCR.
- The NaGene assay is partially inhibited by the currently used GLY transport medium compared to the dedicated Preservation Buffer supplied with the NaGene kit.
- Using the NaGene kit in combination with GLY transport medium for routine screening during the corona outbreak will result in a roughly estimated percentage false negatives of 20-30% in comparison with our in-house qRT-PCR.
- The NaGene PCR assay produced significantly lower CT values (~ 2.7 Ct values) when Preservation Buffer was used as transport medium instead of GLY directly after taking the nasopharyngeal swab.
- In a small comparative study, nasopharyngeal swabs directly stored in Preservation Buffer and analyzed by the NaGene assay resulted in a similar detection rate as compared to the currently used testing strategy with specimens having a Ct up to approx. 34 in our in-house reference assay and 32.6 in the NaGene assay.
- A limitation of this study is the low number of clinical specimens collected in Preservation Buffer and tested in the NaGene assay. To better determine the clinical sensitivity of the NaGene assay especially a higher number of specimens from patients that are borderline positive in our in-house qRT-PCR should be collected in Preservation Buffer and analyzed in the NaGene assay.

Introduction

Routine *in vitro* diagnostics of respiratory RNA viruses is generally performed by means of quantitative reverse-transcription polymerase chain reaction (qRT-PCR) on purified RNA isolated from nasopharyngeal and/or oropharyngeal swabs in a separate process. In contrast, direct PCR is an approach in which the qRT-qPCR is directly performed on the clinical specimen without prior separate extraction and purification of the RNA. These assays may pose a solution to a number of obstacles encountered with the widely used combination of extraction and qRT-PCR. During the initial phase of the SARS-CoV-2 outbreak, a shortage of lysis buffers and consumables for the extraction robots was experienced, which altogether severely reduced testing capacity. In addition, performing the extraction procedures requires additional time and is costly. Lastly, the extraction robots are not or not widely available in low-income countries. Alternatives that circumvent the extraction procedure are much needed. However, direct PCR assays are usually less sensitive than extraction-based protocols, as their efficiency is affected by enzyme inhibitory components present in clinical specimens and because the extraction procedure often includes a concentration step. However, different companies have optimized their enzymes and transport media to improve efficiency. Here, we assessed the sensitivity of the NaGene COVID2019 direct-PCR kit manufactured by NaGene Diagnosis Reagent Co., Ltd. Beijing when used with the standard GLY transport medium or the dedicated NaGene Preservation Buffer (PB) and compared this with that of our currently used extraction-based qRT-PCR testing using dilution series of stored SARS-CoV-2 positive clinical specimens and simulated SARS-CoV-2 clinical specimens. Furthermore, we performed a pilot study with specimens collected from COVID-19 patients in GLY or the NaGene PB followed by the NaGene protocol.

Materials and Methods

The in-house SARS-CoV-2 qRT-PCR was run according to a procedure based on the Corman protocol with primers and probes targeting the E and RdRp genes (1). Briefly, viral RNA was extracted and purified from 200 µl clinical specimen using the MagNa Pure 96 system (Roche); 200 µl specimen is mixed with 275 µl MP lysis buffer with EAV extraction and inhibition control and tRNA stabilizer after which 450 µl is used for extraction and elution in 50 µl of which 5 µl (equivalent to 18.95 µl specimen) is used in the qRT-PCR. The direct NaGene qRT-PCR targeting the ORF1ab and N genes of SARS-CoV-2 was performed according to the manufacturer's instructions with 10 µl clinical specimen in GLY or NaGene PB (at the time of use the protocol did not yet include the vortex and centrifugation step) thoroughly vortexed followed by 2 minute high speed centrifugation step and use of the supernatant in PCR) or purified RNA added to 5 µl NaGene alkaline lysis buffer, incubated for 5 minutes at room temperature after which 35 µl PCR reagents mixture is added containing primers and probes targeting the SARS-CoV-2 ORF1ab and N genes, nucleotides and enzyme. All PCRs were run on a LightCycler 480 II (LC480II, Roche).

GLY transport medium was obtained from Mediaproducts B.V., Groningen, Netherlands. GLY contains components to preserve integrity and infectivity of viruses and prevent overgrowth by bacteria and fungi during transport. Preservation Buffer (PB) was supplied with the NaGene kit. PB contains detergent and betaine and yeast RNA to protect RNA from being degraded by RNAse enzyme.

The specificity of the direct NaGene qRT-PCR was determined by performing the PCR reaction on purified viral RNA of other respiratory (corona)viruses. These included RNA purified from cultured human coronaviruses (CoV) OC43, NL63 and 229E, SARS-CoV, and MERS-CoV and RNA purified from clinical specimens containing influenza virus type A (n=2), RSV-A, RSV-B or rhinovirus (n=2) (prepared and kindly provided by 5.1.2e, Erasmus MC).

To establish PCR efficiency we ran a duplicate 10-fold dilution series of quantified viral RNA (diluted in water with yeast carrier RNA) for both assays. Viral RNA was isolated from SARS-CoV-2 viral particles (hCoV-19/Netherlands/Diemen_1363454/2020, GISAID: EPI_ISL_413570) obtained from cell culture using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). The starting concentration of the viral RNA (copies/µl) was determined by digital PCR targeting the SARS-CoV-2 RdRP-gene and was specific for the positive sense genomic RNA. We determined the slope with R^2 by linear regression in GraphPad Prism and calculated PCR efficiency (E) from the slope. Efficiency was also calculated using dilution series of two clinical specimens; for E-gene in-house qRT-PCR diluted in GLY transport medium and for NaGene assay diluted in NaGene PB. We ran two replicates of a 2-fold dilution series of quantified viral RNA (diluted in yeast carrier RNA in water) to determine the LOD95% by 4-parameter curve fitting with variable slope in GraphPad Prism for NaGene ORF1ab- and N-gene assays and the in-house E-gene qRT-PCR.

Combined nasopharyngeal and oropharyngeal swab specimens (Copan FLOQswabs in 4.5 ml GLY medium) obtained from the different public health services and sentinel general practitioners throughout the Netherlands and stored in the repository of the Centre for Infectious Disease Research, Diagnostics and Laboratory Surveillance (IDS) were used for evaluation whether specimens collected in GLY are compatible with the NaGene kit (n=17).

For the detailed assessment of compatibility of GLY with the NaGene assay clinical samples were 10-fold serially diluted in GLY or PB and subsequently analyzed by the in-house and NaGene assay.

For direct comparison of the influence of the two transport media (GLY and PB) on the performance of the NaGene assay, two nasopharyngeal swabs (Copan FLOQswabs) were taken from 8 hospitalized

COVID-19 patients. One swab taken through the left nostril was collected in 4.5 ml GLY medium and another swab taken through the other nostril was collected in 1 ml PB. All clinical specimens were assessed by both PCR methods once. One patient tested negative in all assays and was excluded from further analysis.

Results

Specificity of NaGene assay

None of the non-coronavirus and non-SARS-COV-2 viral RNAs resulted in a positive signal in the NaGene assay, showing the assay is specific for SARS-CoV-2.

Efficiency and LOD95%

Using previously extracted SARS-CoV-2 RNA directly in the NaGene assay (10 µl) or the in-house E-gene qRT-PCR (5 µl) as reference the LOD95% values for the NaGene N-gene and ORF1ab-gene assays were a factor 10 and 20 higher than the reference (Table 1). Amplification efficiency estimation indicated reduced efficiency compared to that for the in-house E-gene assay, which might have been caused by not including PB in the NaGene qRT-PCR reaction for which it has been optimized. Therefore we analyzed the amplification efficiency using two SARS-CoV-2 positive clinical specimens diluted in PB and subjected to the full NaGene protocol including non-extraction lysis to release virus RNA (Table 1). Also here the NaGene assays showed reduced amplification efficiency compared to the in-house E-gene assay. This does however not necessarily mean reduced clinical sensitivity.

Table 1. Determination of amplification efficiency and LOD95%.

Source material	Specimen diluted in	PCR assay	Efficiency			LOD95% (95% CI) dcopies/ml
			Slope	E	R^2	
SARS-CoV-2 RNA	Water with yeast RNA	NaGene ORF1ab	-2.161	190	0.76	19.3 (15.4-25.6)
		NaGene N	-2.295	173	0.91	8.0 (7.7-8.3)
		E-gene in-house	-3.269	102	0.99	0.92 (0.74-1.2)
SARS-CoV-2 positive clinical specimen 1	PB	NaGene ORF1ab	-2.189	186	0.98	NA
		NaGene N	-1.901	236	0.97	NA
	GLY, MagNApure extraction	E-gene in-house	-2.893	122	0.99	NA
SARS-CoV-2 positive clinical specimen 2	PB	NaGene ORF1ab	-2.744	128	0.79	NA
		NaGene N	-2.305	172	0.75	NA
	GLY, MagNApure extraction	E-gene in-house	-3.160	107	0.96	NA

PB = NaGene Preservation Buffer; NA = Not Applicable

Performance of NaGene assay with clinical specimens routinely collected in GLY transport medium

To investigate the performance of the NaGene assay with clinical specimens routinely collected and stored in GLY transport medium we compared the positivity rate and Ct values to those for our in-house SARS-CoV-2 RT-PCR from 17 clinical specimens collected in GLY from COVID-19 patients (Figure 1). This was done to learn whether widely used GLY in The Netherlands is compatible with the NaGene assay instead of using the dedicated PB to collect the swab in. We found that the Ct values for the NaGene assay were higher and as a consequence some specimens were incorrectly classified as negative (4 out of 17 negative for both NaGene assay targets). With one exception,

clinical specimens with a Ct value below 31 in the E-gene in-house assay were generally detected in the NaGene assay. However, above this value, the sensitivity of the NaGene assay clearly decreased as only one out of four positive specimens with Ct value above 31 in the E-gene in-house assay was confirmed positive with the NaGene assay. Together this indicates that likely GLY transport medium is not compatible with the NaGene assay, either because the release of RNA in the NaGene lysis buffer is affected or the RT-PCR enzyme is inhibited or both.

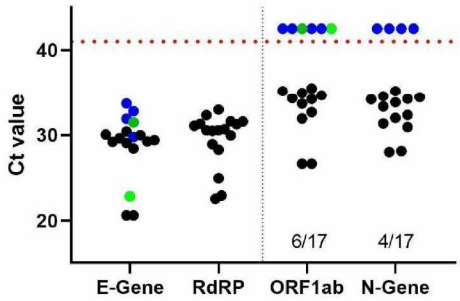

Figure 1. Detection rate of the direct NaGene qRT-PCR assay using clinical specimens collected in GLY transport medium compared with the routinely used in-house qRT-PCR.
Clinical specimens collected in GLY transport medium with a range of Ct values as determined by the in-house SARS-CoV-2 qRT-PCR were used in the NaGene assay to assess its performance. For plotting purposes, negative specimens were set to Ct 42 and plotted above the red dotted line. Blue: not detected in both NaGene assays; Green: not detected in ORF1ab assay. Numbers indicate: negative samples/total samples; the 4 negative for the N-gene target were also negative for the ORF1ab-gene target.

Effect of transport medium on NaGene assay

Efficiency of direct RT-qPCRs may be affected by enzyme inhibiting factors in the specimen of human origin or those present in transport medium. The efficiency of lysis may also be affected by components in the transport medium and therefore a dedicated PB is provided with the NaGene kit. Furthermore, as RNA is not purified the composition of the transport medium can disturb the critical balance of e.g. salts and pH in the PCR reagents mixture in combination with the PB. Since the NaGene assay was provided with a dedicated PB, which inactivates virus and preserves viral RNA, we assessed in more detail what caused the decreased performance of the NaGene kit with the routinely used GLY transport medium as shown with the clinical specimens. For this purpose, we prepared a dilution range of three clinical specimens stored in GLY medium that have a relatively high Ct value to gradually eliminate GLY when diluting in PB. To assess the possible negative effect of transport medium itself, we either diluted in GLY or in PB and determined the Ct value at each dilution in the NaGene assay (Fig. 2). Dilution in GLY medium resulted in loss of signal much more rapidly compared to dilution in PB for both target genes. Using PB, the NaGene assay appears a factor 10 to 1000 more sensitive than when using GLY medium, indicating GLY affected either the lysis or efficiency of the enzyme or both. The N-gene target was most affected by GLY transport medium as in two out of three clinical specimens the N-gene target signal was lost earlier than the ORF1ab signal.

Next, we assessed the relative sensitivities of the NaGene assay and the extraction-based Inhouse RT-PCR. As the negative effect of GLY is gradually removed in a serial dilution in PB this allows for

measuring the relative sensitivity of the direct PCR approach compared with the in-house assay. We compared the Ct values of three clinical specimens with high viral load serially diluted in PB or GLY and then subjected to NaGene or extraction-base qRT-PCR respectively. At low dilutions, the in-house extraction-PCR is more efficient as Ct values are clearly lower (Fig. 2). At higher dilutions, Ct values of the two methods approach each other, indicating diluting out the negative effect of GLY in PB in the NaGene assay. Finally, for two out of the three clinical specimens virus was detected at higher dilutions in the NaGene assay than in the extraction-based in-house qRT-PCR. This indicates the NaGene assay is possibly more sensitive, despite the fact that slightly more specimen equivalent volume is used in the extraction-based in-house qRT-PCR; 10 ul versus 18.95 ul.

These later two experiments show the potency of the NaGene assay, but that it cannot be combined with specimens collected in GLY transport medium. Using specimens collected in PB seems a prerequisite for equal performance with the routinely used extraction-based in-house qRT-PCR protocol.

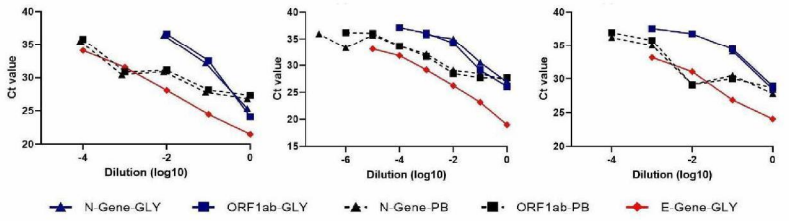

Figure 2. Negative effect of GLY transport medium in NaGene assay and comparison of NaGene assay with extraction-based in-house qRT-PCR.
Three SARS-CoV-2 positive clinical specimens collected in GLY were 10-fold serially diluted in either GLY (blue) or PB (black) as indicated and subsequently assessed for viral RNA by the NaGene assay or the in-house RT-PCR including viral RNA extraction (red). Blue and black lines compare the effect of transport medium and black and red lines compare relative sensitivity of the two strategies; NaGene assay and extraction-based in-house qRT-PCR.

Influence of transport medium on sensitivity of NaGene assay in a clinical setting

Since the negative effect of GLY medium was apparent, we asked whether collection of the swab in the NaGene kits' dedicated Preservation Buffer PB would increase the clinical sensitivity compared to collection in GLY medium. We therefore initiated a small sampling comparison study in which of seven hospitalized COVID-19 patients the nasopharynx was sampled twice, once through the right and once through the left nostril with separate swabs which were collected either in GLY or in PB. Firstly, we investigated if there is an effect of the transport medium on the relative sensitivity (positivity rate) of both assays. For the in-house qRT-PCR, Ct values were similar or even lower when collected directly in PB compared to collected in GLY whereas the positivity rate did not differ (Fig. 3A). For the NaGene assay, all specimens collected in PB resulted in lower Ct values than those obtained upon collection in GLY (Fig. 3A). More importantly, one patient was incorrectly classified as negative in the NaGene assay using the specimen collected in GLY, while SARS-CoV-2 was detected in the PB specimen. Although swabs taken from the same person are compared, the sampling sites might slightly differ, which may result in variation of viral RNA load between swabs. Despite this, the trend is clearly that GLY is not really compatible with the NaGene assay.

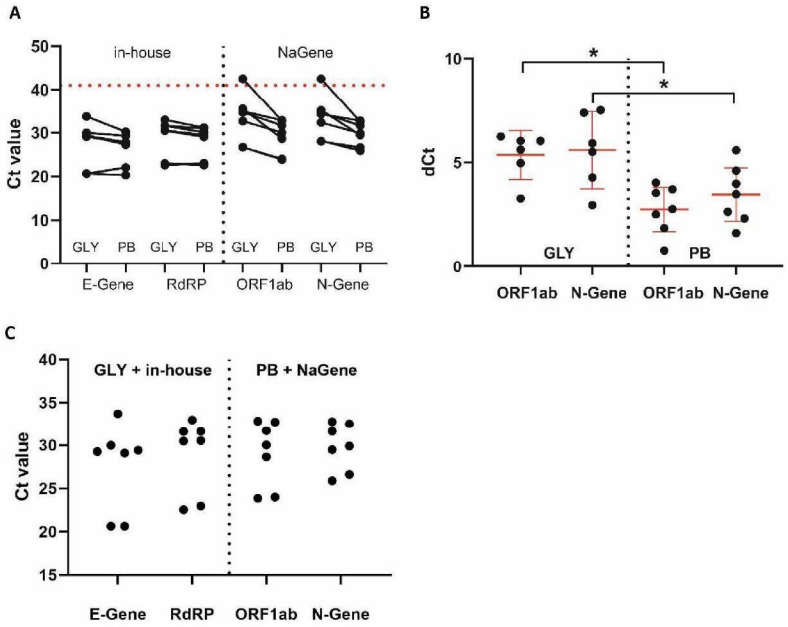

Figure 3. Influence of transport medium on the performance of the NaGene PCR in a clinical setting and Comparison of the extraction-based in-house reference and NaGene strategy

Two nasopharyngeal swabs (through right and left nostril) were taken from seven hospitalized COVID-19 patients and collected in GLY medium or PB. All clinical specimens were analyzed by the in-house and NaGene PCRs. (A) Effect of transport medium in both in-house and NaGene assays. Lines connect swabs taken from the same patient either collected in GLY or PB. (B) Performance of the NaGene assay compared to the in-house E-gene assay in clinical specimens collected in GLY or PB. Presented are the differences in Ct value (dCt) between the in-house E-gene assay and the two NaGene assays performed with the same clinical specimen. The specimen that tested negative by the NaGene assays in GLY was omitted from the dCt calculation. Red bars indicate mean dCt and error bars indicate standard deviation. * $p<0.05$ by a two-tailed Wilcoxon matched-pairs signed rank test. (C) Comparison of the extraction-based in-house reference (collection in GLY medium) and the NaGene strategy (collection in PB and analyzed by the NaGene direct-PCR).

To investigate what the performance of the NaGene assay using PB is compared to the current protocol using extraction-based PCR, we used the most sensitive in-house PCR assay (E-gene) on specimens collected in GLY as a benchmark for comparison using the same specimen. The difference in Ct value between the benchmark and the NaGene assays performed with clinical specimens collected in PB were significantly lower (on average ~2.7 Ct's) than in clinical specimens obtained from the same person but collected in GLY medium (Fig.3B). Moreover, these results show that the NaGene assay provides approximately 2.7 Ct (SD 1.1; ORF1ab) higher Ct values than the in-house assay when swabs are directly collected in PB, whereas this difference is 5.4 Ct (SD 1.1; ORF1ab) for clinical specimens collected in GLY medium. Also these results show that GLY is not really compatible with the NaGene protocol.

Comparison of the current and NaGene testing strategy.

Next, we evaluated how the two PCR strategies directly compare. Thus, the current approach, using GLY transport medium followed by the in-house extraction-based qRT-PCR and collection in PB followed by the NaGene direct-PCR. Figure 3C shows that at higher Ct values all assays resulted in comparable Ct values. Only at lower Ct values the E-gene qRT-PCR generated lower Ct values than the NaGene assays. This is however of less importance in a setting when either a negative or positive result is desired. Interestingly, one specimen with a Ct value of ~34 by the E-gene in-house qRT-PCR was also detected in NaGene assay for both targets, suggesting comparable clinical sensitivity. Together, the results in figure 3 and 4 show that the performance of the NaGene assay is decreased when deviating from the intended protocol by using GLY instead of PB for collection of the clinical specimen. More importantly, the NaGene protocol using specimens collected in PB showed similar performance as our standard extraction-based in-house qRT-PCR protocol.

Prediction range of false negative incidence

To put the observed reduced detection rate when using the NaGene assay using swabs collected in GLY transport medium in perspective, we estimated the percentage false negatives when the NaGene kit would be applied during routine screening in the current coronavirus outbreak. Since we tested only a limited number of clinical samples, we investigated the percentage false negatives of a wide range of Ct values in which possibly the limit of detection of the NaGene kit would fall based on the current evaluation. The database at RIVM containing the outcome of the 'in-house' SARS-CoV-2 qRT-PCR assay on clinical samples obtained from the different public health services during the COVID-19 outbreak in The Netherlands was used to calculate the percentage of SARS-CoV-2 positive specimens below a given Ct value out of the total number of positive specimens (Table 1).

Based on figure 1, the limit of detection of the NaGene assay when performed on clinical specimens collected in GLY medium is predicted to lie between Ct 29-32. Applying the NaGene kit during routine screening in the Netherlands using GLY medium would thus result in an estimated percentage false negative of 20-30% (Table 1). On the other hand, when swabs are directly collected in PB, the NaGene assay detects all specimens tested positive when collected in GLY medium and tested using the standard E-gene in-house qRT-PCR (Figure 3C), including a specimen with Ct ~34 in the in-house E-gene qRT-PCR suggesting full concordance between both approaches. A high number of border-line positive specimens would be needed to determine whether a Ct value can be determined where detection rates start to differ between both approaches.

Table 2: Determination of estimated percentage false negative during routine at range of Ct cutoff values

E-gene in-house RT-PCR Ct value	# below specified Ct	% of SARS-CoV-2 positive	Percentage false negative (%) with detection limit set at Ct in first column
<29	460	70.8	29.2
<30	500	76.9	23.1
<31	521	80.2	19.8
<32	554	85.2	14.8
<33	590	90.8	9.2
<34	623	95.8	4.2
<35	647	99.5	0.5
total # clinical specimens:	2388		
# SARS-CoV-2 positive	650		

Clinical specimens were obtained from different public health services during the COVID-19 outbreak in the Netherlands. For each indicated Ct value (E-gene in-house RT-PCR), the number of SARS-CoV-2 positive specimens is displayed and the percentage of the total number of positive specimens is calculated. Percentage false negative was calculated as the percentage of PCR-positive specimens not detected by the NaGene direct qRT-PCR kit taking the indicated Ct value as cutoff.

Conclusion

Direct RT-qPCR may pose an attractive alternative to current extraction-based qRT-PCR used for laboratory confirmation of SARS-CoV-2 infection. A prerequisite for the application of direct qRT-PCR is that the sensitivity is not hampered too much as direct PCRs may be sensitive to inhibitory components of human origin or those present in transport medium. To prevent the latter the NaGene COVID-2019 PCR kit includes a dedicated collection and transport medium the Preservation Buffer (PB). NaGene therefore recommends to evaluate other transport media before being used, or to used purified RNA (removing however the benefit of using a non-extraction protocol). Our evaluation showed clearly that if GLY transport medium is used instead of PB the sensitivity of NaGene RT-PCR is reduced. When we diluted out the standard used GLY transport medium in a SARS-CoV-2 positive clinical specimen with PB, virus was detected at lower dilutions compared to dilution in GLY transport tmedium. As a result, clinical specimens in GLY with a Ct value above 31 as determined by the in-house SARS-CoV-2 qRT-PCR were incorrectly classified negative using the NaGene kit. Therefore, when the NaGene kit would be used during routine screening with the standard procedure of specimens collected in GLY transport medium in the Netherlands during the coronavirus outbreak this would result in an approximate percentage false negatives of 20-30%. Hence, NaGene non-extraction protocol is not suitable to be used with the current practice of collecting specimens in GLY transportmedium in The Netherlands.

To evaluate if collection of specimens in NaGene PB indeed improves the detection rate, a small sampling study was conducted in which we compared the influence of the two transport media in a clinical setting. In nasopharyngeal swab specimens obtained from hospitalized COVID-19 patients, Ct values were lower for the clinical specimens stored in PB compared to another clinical specimen taken from the same patient but collected in GLY medium. Collection in PB also significantly increased the sensitivity compared to the benchmark in-house E-gene qRT-PCR using GLY transport medium. The average Ct difference between E-gene using GLY specimen and NaGene within the same patient was 2.7 (SD 1.1) when the specimen was collected in PB whereas when collected in GLY transport medium the difference was 5.4 Ct (SD 1.1). Thus when considering implementation of the NaGene kit, it would be strongly advised to also implement collection of the swabs in PB. A

direct comparison of the current testing strategy with the NaGene strategy including the use of PB showed that there were no remarkable differences between the two strategies. Although a samples with a high Ct (34) in the E-gene assay was also detected in the NaGene assay, more samples in this Ct range are required to better define the performance of the NaGene assay compared to the current approach with boarder-line positive specimens.

Logistically it may be a challenge to change the transport medium in routine diagnostics in The Netherlands. One benefit is that we here showed also that the extraction-based qRT-PCR is not affected by PB and therefore when GLY medium was replaced by PB both extraction-based PCR and the NaGene non-extraction protocol could be used.

In general, one should be cautious when applying the NaGene kit using other transport media, as is also outlined in the kit insert to evaluate first compatibility before being used. At least GLY transport medium appears to affect the sensitivity. However, in combination with the supplied Preservation Buffer the NaGene kit is an attractive alternative to extraction-based qRT-PCR for SARS-CoV-2 detection. Before being implemented for high-throughput use few issues should be addressed like centrifugation capacity and automation of the pipetting steps for adding specimen and the reagents mixture.

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Supplementary table 1. Specificity of the NaGene kit

RNA from clinical specimens positive for indicated pathogen	NaGene	Pathogen-specific Ct
Influenza virus type A	neg	26.82
Influenza virus type A	neg	16.81
RSV A	neg	29.24
RSV B	neg	20.02
Rhinovirus	neg	16.64
Rhinovirus	neg	25.9
Negative control	neg	-
Negative control	neg	-
Human coronavirus viral RNA from cell culture		
HCoV OC43	neg	31.67
HCoV NL63	neg	27.2
SARS-CoV-1	neg	21.6
MERS-CoV	neg	34.16
HCoV 229E	neg	33.18