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External Quality Assessment of laboratories Performing SARS-CoV-2 Diagnostics for the Dutch Population, March 2022

Colophon

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Summary

Background

Since January 2020 different workflows for molecular diagnostics of SARS-CoV-2 were implemented and checked for performance using specificity and sensitivity panels distributed by the National Institute for Public Health and the Environment (RIVM). A National External Quality Assessment (EQA) (Landelijk EQA; LEQA) program was developed and implemented by the Dutch COVID-19 WHO reference laboratories at RIVM and Erasmus Medical Centre (Erasmus MC) and the Dutch Ministry of Health, Welfare and Sport ('Dienst Testen'). Main goal is to regularly check the performance of the network of COVID-19 molecular diagnostic laboratories simultaneously. This is also important because of heterogeneity in panels used to assess specificity and sensitivity panels of SARS-CoV-2 Nucleic Acid Amplification Tests (NAAT) performance over time, and the fact that these quality checks were implemented only when workflows changed or laboratories were added to the network.

Since the start of this program five rounds of LEQA have been performed. This report describes the results of the fifth round of the LEQA program.

Objective

The goal of this LEQA round is to assess the quality of the Dutch SARS-CoV-2 molecular diagnostics field, using a panel that consists of 10 simulated clinical specimens, containing heat inactivated SARS-CoV-2 (Delta variant, Omicron BA.1 and Omicron BA.2 variant), SARS-CoV-2 and influenza virus, or hCoV-OC43, or no virus. Each of the laboratories was asked to conduct molecular detection of SARS-CoV-2 according to their workflows used for SARS-CoV-2 diagnostics. Of these 10 specimens, 9 were considered core specimen and one was considered an educational specimen, containing low viral load.

Materials and Methods

In September 2021 and February 2022 the LEQA panel was produced at RIVM. The panel was pretested at RIVM and Erasmus MC in March 2022. Subsequently, all laboratories performing SARS-CoV-2 diagnostics in the Dutch network were contacted and notified of the distribution of the panel in the third week of March 2022. Laboratories were asked to report their results via an online form. Laboratories that perform molecular point of care tests (mPOCT) or other workflows which includes expensive cartridges or pouches, were given the option to test a limited panel consisting of specimens 2, 3, 4, 9 and 10 to reduce costs.

Workflows were given a maximum score of 9 for 100% correct results for the 9 core specimens. The score was reduced by 1 point per specimen for a false positive/negative result and 0.5 points for a result reported as "inconclusive". When a workflow tested the reduced panel (containing specimen 2, 3, 4, 9 and 10), 2 points for a false positive/negative result and 1 point for a result reported as "inconclusive" were subtracted from the maximum score.

Results

Out of 188 workflows reported by 77 participating laboratories, 159 (85.0%) scored a 100% correct score for all 9 core specimens (9 points) and thus met all criteria set for reliable SARS-CoV-2 diagnostics, 20 (10.7%) scored between 8-8.5 points, making it likely that only minor adjustments need to be made to meet all criteria and 9 (4.8%) workflows scored below 8 points. A score below 8 points indicates that improvements need to be made for a workflow to be reliable for SARS-CoV-2 diagnostics. The laboratories that had workflows scoring below 8 points have been contacted individually.

For the core specimens that do not contain SARS-CoV-2 only five (1.5%) false positive or inconclusive results were reported, confirming specificity for SARS-CoV-2 of the vast majority of tested workflows. For the 7 SARS-CoV-2 containing core specimens, 30 false negative results and six inconclusive results

(3.1%) were reported. Despite the wide variety of kits, equipment and enzymes that are used in the different implemented workflows nationwide, the influence of these variations on the overall quality of molecular diagnostics for SARS-CoV-2 was limited. Additionally, SARS-CoV-2 variants incorporated in the panel were detected equally well in the included workflows.

Conclusions

Overall, the workflows used for SARS-CoV-2 diagnostics perform very well and laboratories using them provide a reliable network. A small number of workflows should be further optimized to achieve full potential. The Dutch SARS-CoV-2 diagnostics laboratory network performs on a very high level with the vast majority of workflows detecting the core SARS-CoV-2 containing specimens correctly. The wide variety of kits, equipment and enzymes used in the Dutch SARS-CoV-2 diagnostic field do not affect adversely the quality of diagnostics. Instead, it allows for greater flexibility during times of shortages in supplies and likely improves the capacity to detect possible future variants of SARS-CoV-2. In addition it reduces the likelihood of a collapse of the network if a particular assay fails due to evolution of the virus as different workflows make use of different techniques and primer/probe combinations.

1. Introduction

Since January 2020 a wide variety of workflows for molecular diagnostics of COVID-19 were implemented and checked for performance using specificity and sensitivity panels distributed by the National Institute for Public Health and the Environment (RIVM). Although panels have been largely similar in viral load components for checking SARS-CoV-2 Nucleic Acid Amplification Tests (NAAT) performance, they initially contained SARS-CoV-1 RNA, later replaced by SARS-CoV-2 RNA, followed by SARS-CoV-2 whole heat inactivated virus particles, depending on when materials became available. Because of this heterogeneity in the past, the fact that patchy quality checks were implemented only when workflows changed or laboratories were added to the network, and because it is important to regularly check the performance of the COVID-19 molecular diagnostic lab network, the COVID-19 WHO reference laboratories at RIVM and Erasmus Medical Centre (Erasmus MC) and the Dutch Ministry of Health, Welfare and Sport ('Dienst Testen') have developed a National External Quality Assessment (EQA) (Landelijk EQA; LEQA) program.

In the third week of March 2022 the fifth round of EQA panels was distributed to all laboratories performing SARS-CoV-2 diagnostics on clinical specimens derived from Dutch patients. This panel consisted of 10 simulated clinical specimens that contained either heat inactivated SARS-CoV-2 (Delta variant, Omicron BA.1 and Omicron BA.2 variant), live hCoV-OC34, a combination of SARS-CoV-2 and live influenza A(H3N2) or no virus. Each of the laboratories was asked to conduct molecular detection of SARS-CoV-2 on this panel according to their workflows normally used for SARS-CoV-2 diagnostics. All data had to be reported back to the RIVM using an online reporting form.

2 Materials and methods

2.1 Approach

The LEQA panel was produced at the RIVM in September 2021 and February 2022. The panel was pretested at RIVM and Erasmus MC in March 2022. Both centers obtained similar results, allowing distribution of the panel. The laboratories performing SARS-CoV-2 diagnostics in the Dutch network were contacted and notified of the distribution of the panel planned in the third week of March 2022. All laboratories were asked to test and voluntarily report their findings via an online form using Formdesk software (Wassenaar, The Netherlands). Laboratories were given time until the 3rd of April 2022 to report their obtained results. A number of workflows, especially the molecular point of care (mPOCT) ones, use expensive cartridges or pouches. Therefore laboratories that wanted to test these workflows with a limited panel were given the option to do so. The reduced panel consisted of specimens 2, 3, 4, 9 and 10 (Table 1), covering one concentration of SARS-CoV-2 Omicron BA.1 variant, two different concentrations of SARS-CoV-2 Omicron BA.2 variant, a SARS-CoV-2 Delta variant and influenza virus A(H3N2) double positive specimen and a no virus control.

2.2 Contents of LEQA5 panel

The LEQA5 panel consisted of 10 simulated clinical specimens (1ml) containing either whole infectious human respiratory seasonal viruses influenza virus A(H3N2) or hCoV-OC43, heat-inactivated SARS-CoV-2 viruses (SARS-CoV-2 Omicron BA.1 or BA.2 variant) or no virus. One specimen contained both SARS-CoV-2 Delta variant and influenza virus. SARS-CoV-2 was isolated from clinical specimens on VERO E6 cells and inactivated by heat treatment at 60 °C for two hours. The number of detectable copies of SARS-CoV-2 positive strand RNA in the stocks of SARS-CoV-2 was back-calculated from determination of the copy number after extraction of RNA by digital SARS-CoV-2 E-gene and RdRP-gene PCR reference. Because the viruses were not purified from the supernatant, the whole virus preparation contains in addition to genomic RNA, intermediate replication negative strand genomic

RNA and subgenomic messenger RNAs that contribute to detection in routine one-step RT-qPCR for SARS-CoV-2 RNA. Virus dilutions were made in MEM with Hanks' salts. HEp2 cells were added to the dilution at a concentration of 10.000 cells per ml panel specimen to simulate a clinical specimen. The 10 specimens included in the panel contained the following viruses: SARS-CoV-2 Delta (variant B.1.617.2;hCoV-19/Netherlands/NH-RIVM-27142/2021) in one concentration, SARS-CoV-2 Omicron BA.1 (hCoV-19/Netherlands/NH-RIVM-71076/2021) SARS-CoV-2 Omicron BA.2 (hCoV-19/Netherlands/ZH-RIVM-83862/2022), hCoV-OC43 (ATCC), influenza virus A(H3N2) (A/Netherlands/10002/2019) and a specimen without any virus. Seasonal hCoV-OC43 and influenza A(H3N2) viruses were not inactivated. In Table 1 all specimens are listed together with the expected target specific Cq values obtained at RIVM with routinely used diagnostic RT-qPCRs for the respective pathogens and the expected conclusion for SARS-CoV-2 detection in the specimens. The digital copies of RdRP-gene and E-gene are also listed in Table 1 for the SARS-CoV-2 containing specimens.

Table 1: Composition of the LEQA5 panel and its target specific expected Cq values[1] based on the in-house assay(s) of the RIVM.

Panel coding	Virus[2]	Number of copies E gene target/ml specimen, determined with dPCR[3]	Number of copies RdRP gene target/ml specimen, determined with dPCR[3]	Target specific Cq[4]	E-gene (Sarbeco) Cq	RdRP-gene (SARS-CoV-2) Cq	Conclusion SARS-CoV-2
LEQA5_CoV21-1	SARS-CoV-2 BA.2 (d2)	32046	17300	-	31.33 (4/4)	31.93 (4/4)	POSITIVE
LEQA5_CoV21-2	SARS-CoV-2 BA.2 (d3)	3205	1730	-	33.99 (4/4)	34.87 (4/4)	POSITIVE
LEQA5_CoV21-3[5]	Influenza A(H3N2) + SARS-CoV-2 Delta	550000	394000	34.25 (4/4)	28.20 (4/4)	28.64 (4/4)	POSITIVE
LEQA5_CoV21-4	SARS-CoV-2 BA.1 (d2)	19921	17300	-	33.35 (4/4)	33.26 (4/4)	POSITIVE
LEQA5_CoV21-5	SARS-CoV-2 BA.2 (d1)	320456	173000	-	27.52 (4/4)	28.11 (4/4)	POSITIVE
LEQA5_CoV21-6	SARS-CoV-2 BA.1 (d1)	199200	173000	-	29.24 (4/4)	29.75 (4/4)	POSITIVE
LEQA5_CoV21-7	SARS-CoV-2 BA.2 (d3)	3205	1730	-	33.11 (4/4)	34.85 (4/4)	POSITIVE
LEQA5_CoV21-8	hCoV-OC43	-	-	27.68 (4/4)	-	-	Negative
LEQA5_CoV21-9	No virus	-	-	-	-	-	Negative
LEQA5_CoV21-10[6]	SARS-CoV-2 BA.2 (d4)	320	173	-	34.74 (1/4)	36.20 (3/4)	Weakly POSITIVE

[1] The expected Cq values shown in this table are based on RT-qPCR tests performed on the panel specimens using the routinely used RIVM in-house assays. The in-house real-time RT-qPCRs have been performed using the following reagents and volumes: ThermoFisher TaqMan® Fast Virus 1-Step Master Mix after extraction of 200 µl specimen on Roche MagNA Pure 96 instrument with Roche MagNA Pure 96 DNA and Viral NA Small Volume Kit, elution in 50 µl and 5 µl extract per RT-qPCR reaction on Roche LightCycler 480 mark I or II. Extractions and subsequent RT-qPCRs were performed in 4-fold; after the average Cq value between brackets () the number of times found positive is shown. SARS-CoV-2 E-gene Sarbeco specific primers and probes are those published by Corman *et al.* 2020; the RdRP primers and probes are modified from those published by Corman *et al.* (2020) to become SARS-CoV-2 specific and similar in LOD95 compared to the E-gene Rt-qPCR.

[2] d1, d2, d3 and d4 indicate that these specimens are a 1:10 dilution series. Dilution d1 has the highest concentration of SARS-CoV-2 and d4 the lowest. SARS-CoV-2 is heat inactivated.

[3] dPCR has been performed on + strand genomic RNA for RdRP-gene and E-gene. The one-step E-gene and RdRP-gene diagnostic RT-qPCR also detects - strand replicative form genomic RNA and the one-step E gene RT-qPCR in addition also detects subgenomic messengers, which probably increases the actual number of target templates for the diagnostic RT-qPCR in the specimen after extraction.

[4] For hCoV-OC43 N-gene; for influenza virus M-gene

[5] Double positive specimen for both SARS-CoV-2 Delta variant and influenza A(H3N2) virus. Contained a high copy number of SARS-CoV-2 and low copy number of A(H3N2) to be able to detect possible interference. Detection of influenza virus in this specimen is educational.

[6] Educational specimen: repeats of this specimen may have the E-gene and/or RdRP-gene negative at the reference laboratory; in general, only 62.4% of the workflows reported this specimen positive for SARS-CoV-2

2.3 Scoring the workflows

Each reported workflow was scored on a scale from 0 to 9 (maximum score), based on the 9 core specimens which contained clinically relevant amounts of virus or no virus. The educational specimen LEQA5_CoV21-10 was not deemed a core specimen. The laboratories were given the option to evaluate specimens with the following scores: positive, negative or inconclusive. The term "inconclusive" was used to indicate uncertainty concerning the test result(s) obtained.

Each workflow started with a total of 9 points. For each wrongly determined core specimen (being positive for a specimen containing no SARS-CoV-2 or vice versa) 1 point was deducted (out of 9). When a core specimen was scored with an "inconclusive" result, 0.5 point was deducted from the final mark of the workflow.

For some workflows (e.g., molecular point-of-care test (mPOCT) workflows) an option was given to test a smaller subset of specimens to enable making a limited statement about the sensitivity of detection of SARS-CoV-2 with the mPOCT used. These workflows only had to test LEQA5_CoV21-2, LEQA5_CoV21-3, LEQA5_CoV21-4, LEQA5_CoV21-9 and LEQA5_CoV21-10. The workflows testing the reduced panel were scored according to a scale from 1 – 9 points. For each wrongly determined core specimen (being positive for a specimen containing no SARS-CoV-2 or vice versa) 2 points were deducted (out of 9). When a specimen was scored with an "inconclusive" result, 1 point was deducted from the final mark of the workflow.

A workflow scoring 9 out of 9 passed all criteria set for SARS-CoV-2 diagnostics in terms of sensitivity and specificity deemed necessary for SARS-CoV-2 diagnostics in accordance with the set requirements for new workflows and laboratories. [1] Workflows scoring 8 or 8.5 out of 9 need some adjustment(s) to perform as desired. Adjustments depend on the type of result, e.g. an "inconclusive" result for low viral load LEQA5_CoV21-02, LEQA5_CoV21-04 LEQA5_CoV21-07 specimens is less severe than detection of SARS-CoV-2 targets in specimens which do not contain SARS-CoV-2 (false positive). Any workflow scoring below 8 out of 9 points needs serious adjustments in order to be fit for SARS-CoV-2 diagnostics.

3. Results

3.1 Aggregated overview

Eighty-five laboratories were contacted with the announcement of panel distribution for this fourth EQA round. Seventy-seven (90.6%) of these laboratories reported their findings for a total of 189 workflows (participating laboratories summarized in Supplement 6.5). One dataset was submitted twice by accident. One of these two datasets was removed. The remaining 188 datasets were analyzed. The workflow results reported for each panel specimen are summarized in Table 2. The panel scores obtained per laboratory and by number of workflows used are summarized in Table 3. Despite not all workflows obtaining fully correct results with the core specimens (Table 2), nearly all laboratories (76/77; 98.8%) used at least one workflow for which a score of 8 to 9 was obtained (Table 3)**.** More detailed information on performances of workflows per laboratory is shown in Supplemental Figure 1. No difference in detectability was seen for the included SARS-CoV-2 variants. Using a heatmap, Figure 1 shows a summary of the overall results obtained by all workflows. In the subsequent chapters a more detailed insight into the results and their background is presented.

Table 2. Aggregated overview of workflow conclusions by LEQA5 panel specimen.

Panel specimen	Content	№ of workflows with test result reported (n=188)[1]	SARS-CoV-2 detection workflow conclusion			
			№ Positive	№ Negative	№ Inconclusive	Errors
LEQA5_CoV21-9	No virus	188	1	187	0	False positive result (n=1)
LEQA5_CoV21-8	hCoV-OC43	147	3	143	1	False positive result (n=3); Inconclusive result (n=1)
LEQA5_CoV21-3	Influenza A(H3N2) + SARS-CoV-2 Delta	188	188	0	0	None
LEQA5_CoV21-6	SARS-CoV-2 BA.1 (d1)	147	146	1	0	False negative result (n=1)
LEQA5_CoV21-4	SARS-CoV-2 BA.1 (d2)	188	182	5	1	False negative result (n=5); Inconclusive result (n=1)
LEQA5_CoV21-5	SARS-CoV-2 BA.2 (d1)	147	147	0	0	None
LEQA5_CoV21-1	SARS-CoV-2 BA.2 (d2)	147	146	0	1	Inconclusive result (n=1)
LEQA5_CoV21-2	SARS-CoV-2 BA.2 (d3)	188	173	12	3	False negative result (n=12); Inconclusive result (n=3)
LEQA5_CoV21-7	SARS-CoV-2 BA.2 (d3)	147	134	12	1	False negative result (n=12); Inconclusive result (n=1)
LEQA5_CoV21-10	SARS-CoV-2 BA.2 (d4)	188	118	63	7	Not applicable, educational specimen

[1] 41 of the 188 workflows tested were mPOCT testing specimens 2, 3, 4, 9 and 10 only. Therefore, the number of workflows with test result per specimen is 147 for specimen 1, 5, 6, 7 and 8.

Table 3. Aggregated overview of scores for core specimens obtained by laboratories using various numbers of workflows.

№ of workflows per lab	№ of labs	№ of workflows per lab with indicated score (No of labs)		
		Score 9	Score 8.5 or 8	Score < 8
7	2	3-5 (n=2)	2-3 (n=2)	1 (n=1)
6	3	5-6 (n=3)	1 (n=2)	-
5	2	4-5 (n=2)	-	1 (n=1)
4	13	3-4 (n=13)	1 (n=5)	1 (n=1)
3	11	2-3 (n=11)	1 (n=2)	1 (n=3)
2	15	1-2 (n=15)	1 (n=4)	1 (n=2)
1	31	1 (n=28)	1 (n=2)	1 (n=1)

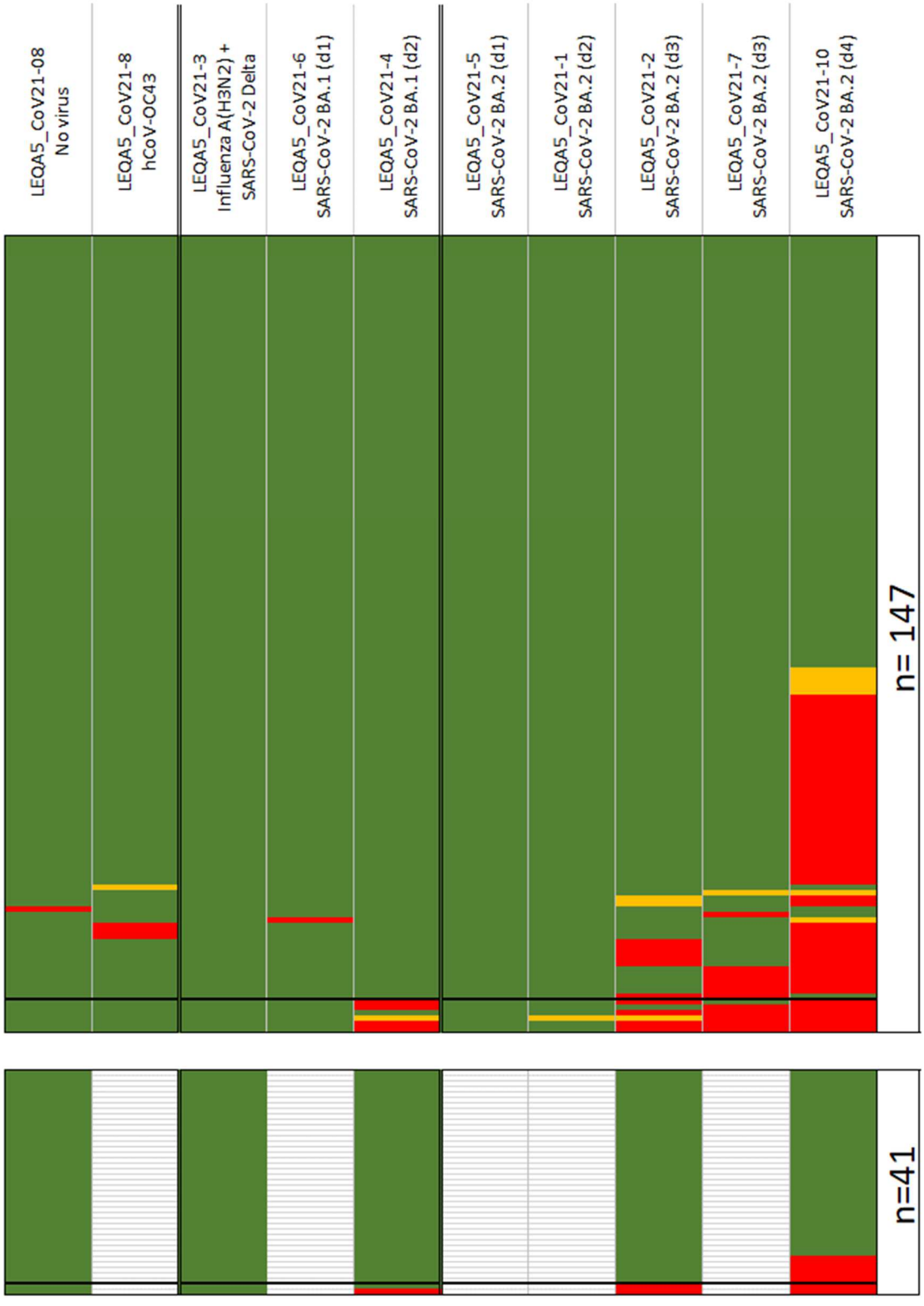

Figure 1: A heatmap showing all final conclusions per sample per workflow submitted. Green blocks represent a correct result, red blocks indicate an incorrect result and orange blocks display inconclusive results. The workflows displayed in this heatmap are sorted by the obtained scores. The thick horizontal black line in the top segment of the figure separates all workflows scoring 8-9 points and all workflows scoring <8 points. All workflows testing the reduced panel (n=41) are displayed in the lower segment of the figure (and are sorted by score). When a box remains white, this sample was not tested by that particular workflow as it tested the reduced panel The thick horizontal black line in the lower segment of the figure separates all workflows scoring 8-9 points and the workflow scoring <8 points. All laboratories using a workflow scoring <8 points have been contacted. All samples are arranged similarly as in Table 2. As LEQA5_CoV21-10 is an educational sample with a very low viral load, many workflows failed to detect SARS-CoV-2 in this sample.

3.2 Target genes used for RT-PCR or other NAAT

For all workflows the target genes used were inventoried based on the online report form. Workflows used up to 4 target genes. Table 4 shows the scores obtained by all workflows testing LEQA5 sorted by the number of target genes used. From the 188 workflows a total of 55 workflows used 1 target gene, 97 workflows used 2 target genes, 32 workflows used 3 target genes and 4 workflows used 4 target genes. Some workflows using more than one target gene do not generate separate result for each independent gene but rather a composite conclusion. The exact combinations of target genes used in the included workflows are shown in Supplemental Table 1.

Table 4. Scores obtained by all workflows sorted by number of target genes used

№ of target genes per workflow	№ of workflows	№ of workflows with indicated score		
		Score 9	Score 8 or 8.5	Score < 8
1	55	42 (76.4%)	8 (14.5%)	5 (9.1%)
2	97	84 (86.6%)	9 (6.1%)	4 (4.1%)
3	32	30 (93.8%)	2 (6.3%)	0
4	4	3 (75.00%)	1 (25.00%)	0

3.3 Performance of the workflows

As described before, all workflows were graded using a point system from 0 (being the lowest grade) up to 9 (highest grade). One point was given for each of the core specimen in the complete panel. Two points for each of the core specimen was given when testing the reduced (mPOCT) panel. In total 159 workflows were given a '9', four workflows scored an '8.5', 16 workflows scored an '8', five workflows scored a '7', one workflow scored a '6.5', two workflows scored a '6' and one workflow scored a '5'. Figure 2 shows all grades given to the reported workflows.

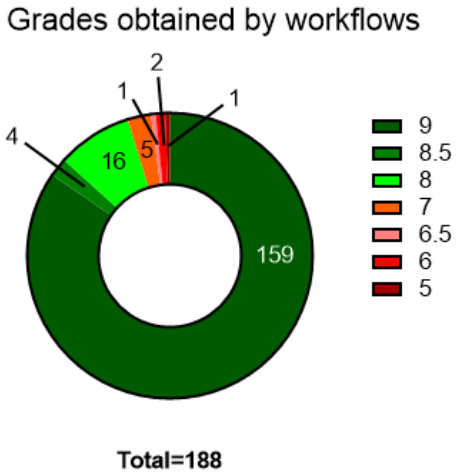

Figure 2: All grades obtained by the reported workflows out of the maximum of 9 points (n=188).

A wide spread in reported Cq values was seen for the specimens of the panel. The biggest range of reported Cq values was found for LEQA5_CoV21-3 for target gene E-gene Sarbeco specific. The average Cq value for E-gene Sarbeco specific was 28.6 (range Cq 18.7 – 34.9, median 28.0). This indicates a wide spread of Cq values reported for the same specimen, generated by the large variety of workflows used. Despite that, generally the difference in Cq values within a workflow was proportional to the viral load for all variants included. An overview containing the results obtained per target gene per specimen for workflows reporting Cq values is shown in Supplemental Figures 1 and 2. In these figures for each of the target genes used the Cq values are shown for each of the tested specimens.

The obtained scores per workflow are also linked to the used extraction kit or method, the performed PCR or other NAAT in order to assess the effect of different techniques on the performance of workflows. Some RNA isolation (13/50; 26%) and RNA amplification (13/50; 26%) techniques performed below average (≥50% of the obtained scores below 9). In total these below average performing techniques were implemented in 27/188 (14.4%) of the cases for RNA isolation and 29/188 (15.4%) of the cases for RNA amplification. Fortunately very few RNA isolation and RNA amplification techniques showed poor overall performance when combined. Generally all implemented techniques showed the capability to perform at a high level when implemented correctly. An overview of the scores obtained with different combinations of implemented techniques is shown in Supplemental Figure 3. Summaries of kits, equipment and/or separate enzymes used in the submitted workflows are shown in Supplemental Figure 5-9.

4. Discussion and conclusion

Here we describe the results of the fifth national LEQA panel. 77 laboratories, using in total 189 workflows have reported their findings; one duplicate report was removed from analysis. Out of the 188 workflows analyzed, 159/188 (85.0%) scored a maximum score for all 9 core specimens (9 points) and thus met all criteria set for reliable SARS-CoV-2 diagnostics. 20/188 (10.7%) scored between 8.5-8, making it likely that only minor adjustments need to be made to meet all criteria and 8/189 (4.3%) workflows scored a 7.5 or below. When scoring below 8 points, it is an indication that major improvements need to be made for these workflows to be reliable for SARS-CoV-2 diagnostics in clinical diagnostic settings and surveillance. All laboratories with workflows scoring below 8 points have been contacted and have been given recommendations how to improve their performance.

For the seven SARS-CoV-2 containing core specimens, 30 false negative results and 6 inconclusive results were reported; 3.1% of total reported results. Four false positive and 1 inconclusive results were reported for the two specimens without SARS-CoV-2; 1.5% of total reported results. Sensitivity seems a bigger issue than specificity for the workflows used for SARS-CoV-2 diagnostics, although the difference was not statistical significant (Z-test 2-tailed p=0.1074 at 95% confidence). All laboratories reporting false positive and inconclusive results for specimens not containing SARS-CoV-2 have been contacted. One laboratory does not have any workflows scoring an 8 higher, which has been contacted and given recommendations on how to improve their performance.

When comparing all workflows, there appears to be a slight correlation between the number of target genes used and the score obtained by the workflow. The average scores obtained are 8.64 (n=56), 8.81 (n=97), 8.95 (n=32) and 8.75 (n=4) out of 9 for workflows containing one, two, three and four target genes, respectively. The median score was 9 for all four types of workflows. Workflows with 3 target genes appear to have the highest average score. This dataset is, however, skewed due to one PCR-kit (Cepheid, Xpert® Xpress CoV-2/Flu/RSV plus) involved in 25/33 assays in this group (with 3 target genes). An argument in favor of multiple target genes is that it decreases the chance of false negative test results when testing (novel) SARS-CoV-2 variants. Mutations in (novel) SARS-CoV-2 variants might cause a target gene dropout in assays. Also other mutations are able to render primer/probe combinations less effective in detecting SARS-CoV-2. [2,3] It is possible that other novel SARS-CoV-2 variants cause target gene dropouts as primer/probe sets are not optimized for that particular new genetic variant. If an assay were to use only one target gene, there is a higher chance for a false negative result than in assays with multiple target genes in case of new variants that affect primer or probe target sites. A downside of using multiple target genes is an increased uncertainty in final conclusion when two (or more) target genes show different results.

For all LEQA5 specimens tested with a workflow providing Cq values as an output, a broad range of Cq values for the individual SARS-CoV-2 containing specimens has been reported. Despite this wide range of Cq values for the same specimen by different workflows, it did not seem to affect the sensitivity of the workflows when looking at the reported diagnostic result (positive vs. negative). This finding indicates that comparing Cq values between workflows and laboratories is not possible without prior calibration using a standard.

A wide array of varying in-house and kit-based SARS-CoV-2 workflows have been reported. A more divergent use of kits, reagents and equipment can be quite useful in a laboratory network as a shortage of any of these can be compensated by switching to different equipment or when certain workflows are less capable of detecting new strains of SARS-CoV-2. This is highly relevant with the frequent emergence of novel variants of SARS-CoV-2.

A small part of the workflows assessed need to be improved in order to perform as desired. For these workflows we hope that the laboratories involved will search for possible improvements on their

workflow(s). All in all the Dutch SARS-CoV-2 diagnostics laboratory network appears to perform on a very high level providing accurate SARS-CoV-2 molecular detection services.

5. References

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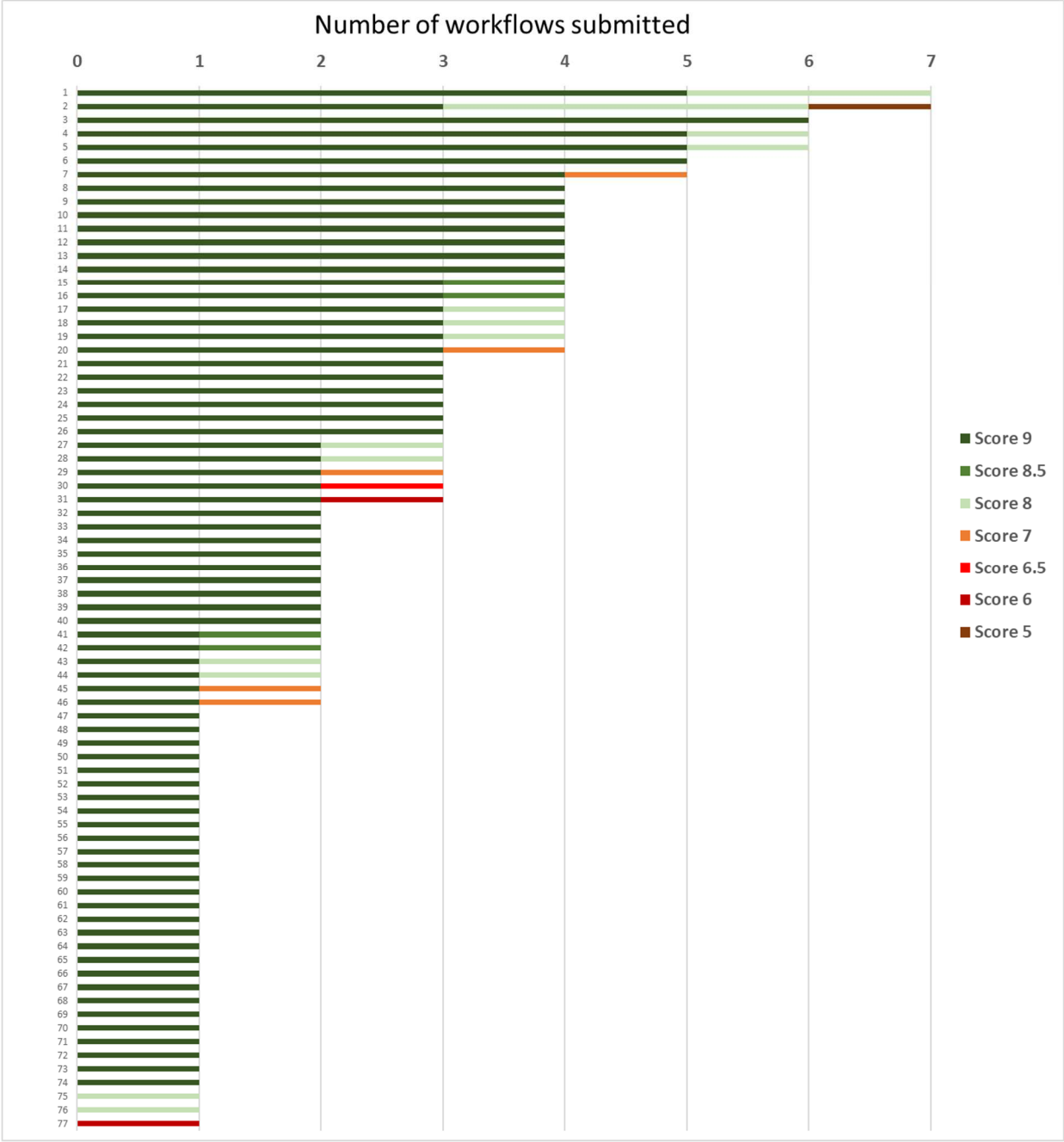
6. Supplemental material

6.1 Scores obtained per laboratory

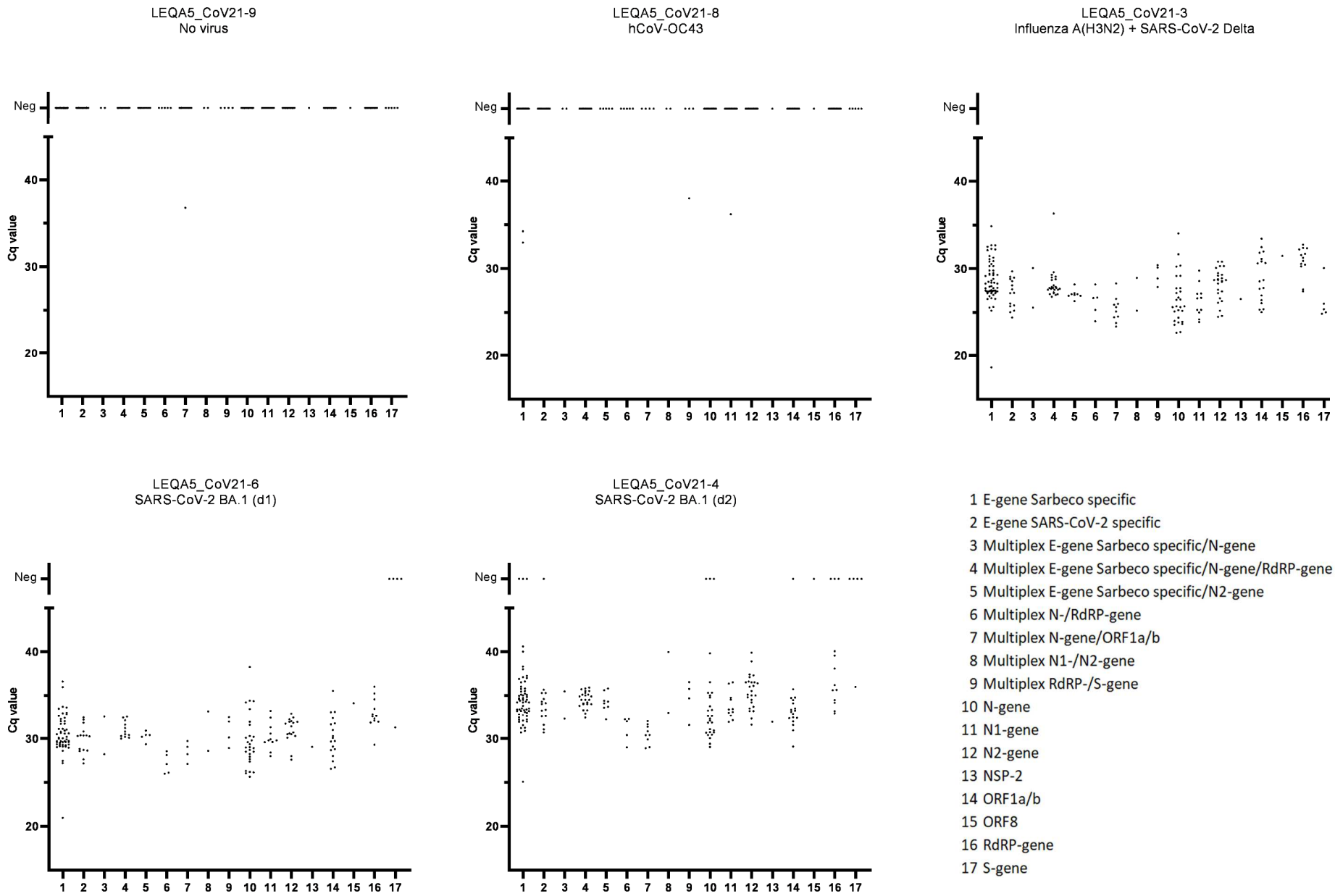
Here all obtained scores per workflow per laboratory are summarized. In total two laboratory reported data for 7 workflows, three laboratories reported data for 6 workflows, two laboratories reported data for 5 workflows, 13 laboratories reported data for 4 workflows, 11 laboratories reported data for 3 workflows, 15 laboratories reported data for 2 workflows and 31 laboratories reported data for 1 workflow. There is one laboratory which only has scores of < 8 for all reported workflows. The obtained scores per workflow are sorted (anonymously) per laboratory and shown in Supplemental Figure 1. Of the 77 laboratories, 74 laboratories reported at least one workflow with fully correct results.



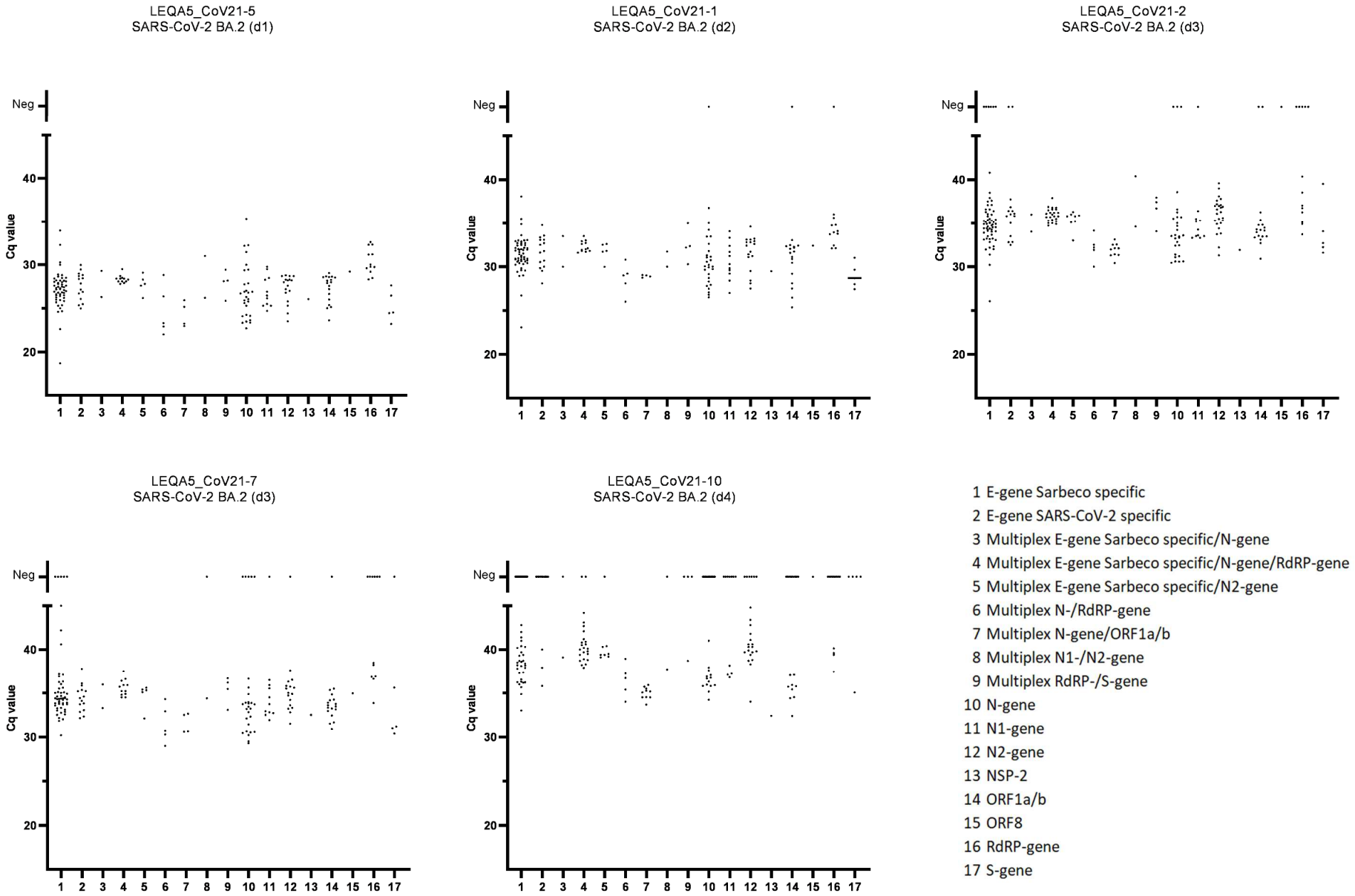
Supplemental Figure 1: Grades obtained per workflow per laboratory (anonymized). For each of the laboratories the number of reported workflows is shown on the X-axis together with their accompanying grades. In total 77 laboratories sent in data of their workflows testing LEQA5. There is one laboratory which only has scores of < 7 for all reported workflows. All laboratories using a workflow scoring <8 points have been contacted

6.2 Cq values obtained per panel specimen per target gene

In total 17 different target genes with Cq values were reported in the submitted workflows. When looking at the Cq values obtained, it seems that using the same target genes can result in big variations even within the same specimen. In Supplemental Figures 2 and 3 the Cq values found for each target gene per specimen are shown.



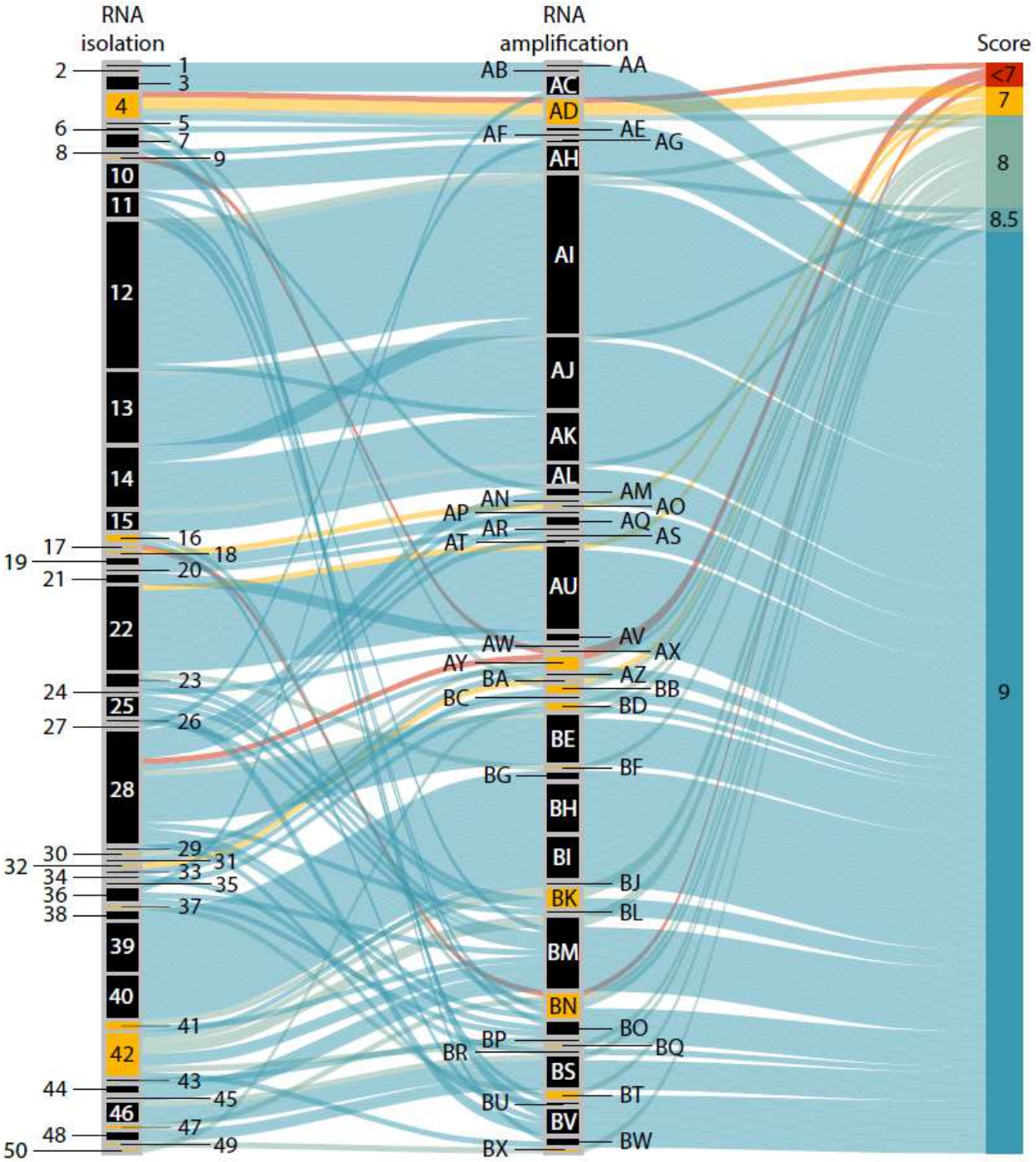
Supplemental Figure 2: All Cq values obtained by Cq value reporting workflows for specimens LEQA5_CoV21-9, LEQA5_CoV21-8, LEQA5_CoV21-3, LEQA5_CoV21-6 and LEQA5_CoV21-4 (same specimen order as in Table 2). In total 17 different target genes were reported with Cq values, some of which were multiplex target genes. Cq values obtained from workflows using multiple individually reported target genes area all reported separately from each other. The legend displayed shows which target genes were used. Negative results are clustered separately above the Y-axis.



Supplemental Figure 3: All Cq values obtained by Cq value reporting workflows for specimens LEQA5_CoV21-5, LEQA5_CoV21-1, LEQA5_CoV21-2, LEQA5_CoV21-7 and LEQA5_CoV21-10 (same specimen order as in Table 2). In total 17 different target genes were reported with Cq values, some of which were multiplex target genes. Cq values obtained from workflows using multiple individually reported target genes area all reported separately from each other. The legend displayed shows which target genes were used. Negative results are clustered separately above the Y-axis.

6.3 Effect of RNA isolation and NAAT reaction on score obtained by workflow

In order to see how well certain combinations of RNA isolation and amplification techniques perform together, an analysis was performed in which these factors were combined with their final score. These data are summarized in Supplemental Figure 4. If for example one type of RNA isolation technique has poor compatibility with a specific RNA amplification technique, you would expect a big band of poor scores flowing through both categories. Poor performing RNA isolation and RNA amplification techniques/kits have been highlighted with yellow.



Supplemental Figure 4: A flow diagram showing all workflows reported to have tested the LEQA5 panel with extraction method, PCR test, the number of target genes used and the final score achieved by each workflow. Color of trails per workflow are based on the grade obtained for LEQA5. All workflows receiving grades below 7 are grouped in <7. Both RNA isolation and RNA amplification kits/techniques with 50% or more scores below 9 have been highlighted with yellow in their respective categories. These techniques show lower performance than other techniques in their respective category used in this EQA round. All laboratories using a workflow scoring <8 points have been contacted

RNA isolation	
1	3DMed, ANDiS Viral Nucleic RNA Auto Extraction & Purification Kit
2	Abbott, Alinity m Resp-4-Plex assay
3	Abbott, ALINITY m SARS-COV-2 ASSAY
4	Abbott, ID NOW™ COVID-19
5	Abclonal, ANDiS Viral Nucleic RNA Auto Extraction & Purification Kit
6	Altona Diagnostics, AltoStar® Purification Kit 1.5
7	Applied Biosystems, MagMAX TM Viral/Pathogen II Nucleic Acid Isolation Kit
8	BD, BD MAX SARS-CoV-2 kit
9	Bioecho, EchoLUTION viral RNA/DNA Swab 96 Kit
10	BioMerieux Diagnostics, Respiratory panel 2.1 plus
11	BioMerieux, NUCLISENS® easyMAG®
12	Cepheid, Xpert® Xpress CoV-2/Flu/RSV plus
13	Cepheid, Xpert® Xpress SARS-CoV-2
14	Cepheid, Xpert® Xpress SARS-CoV-2/Flu/RSV
15	Certest, VIASURE SARS-CoV-2 Real Time PCR
16	Chemagen, Chemagic Viral DNA/RNA 300 kit H96
17	Clean NA, Viral DNA & RNA SP kit
18	ELITech Group, SARS-CoV-2 ELITe MGB® Kit
19	GenMark Diagnostics, ePlex® RP2
20	Goldstandard Diagnostics, NovaPrime RNA Extraction AE1 RTU
21	Hologic, Aptima® SARS CoV2/FLU assay
22	Hologic, Aptima® SARS-CoV-2 Assay
23	Hologic, Panther Fusion® Open Access™
24	Hologic, Panther Fusion® SARS-CoV-2 assay
25	Life Technologies, MagMax pathogen RNA/DNA kit
26	Magcore, MagCore® Viral Nucleic Acid Extraction Kit
27	MGI, MGIEasy Nucleic Acid Extraction Kit
28	Molgen, PurePrep Pathogens
29	NeuMoDx™, Flu A-B/RSV/SARS-CoV-2 Vantage Test Strip
30	Pathofinder, RealAccurate® Quadruplex SARS-CoV-2 PCR Kit
31	PerkinElmer, chemagic Viral DNA/RNA 300 Kit H96
32	PhoenixDx, PDX COVID-19
33	Procomcure Biotech, SphaeraMag DNA/RNA Isolation Kit - prefilled96
34	Promega, Maxwell RSC Viral TNA Kit
35	Qiagen, QIAprep&Amp
36	Qiagen, QIAsymphony DSP Virus/Pathogen Midi Kit
37	Qiagen, QIAsymphony DSP Virus/Pathogen Mini Kit
38	Roche, Cobas® Liat SARS-COV-2
39	Roche, Cobas® Liat SARS-COV-2/FluA/FluB
40	Roche, Cobas® SARS-CoV-2: Qualitative assay for use on the cobas 6800/8800 Systems
41	Roche, DNA and Viral NA Large Volume kit
42	Roche, DNA and Viral NA Small Volume kit
43	Roche, MagNA Pure 24 Total NA Isolation Kit
44	Roche, MagNA Pure 96 Total Nucleic Acid Isolation Kit
45	Roche, MagNA Pure LC Total Nucleic Acid Isolation Kit
46	Seegene, STARMag 96x4 Universal Cartridge Kit
47	Siemens, VERSANT Sample Preparation 1.0 Reagents
48	TANBead, Nucleic Acid Extraction kit
49	Zeesan, lab-aid Virus RNA extraction kit
50	None

RNA amplification	
AA	3DMed, ANDiS Fast SARS-CoV-2 RT-qPCR Detection Kit
AB	Abbott, Alinity m Resp-4-Plex assay
AC	Abbott, ALINITY m SARS-COV-2 ASSAY
AD	Abbott, ID NOW™ COVID-19
AE	Altona Diagnostics, Altona Realstar SARS-CoV-2 RT PCR
AF	BD, SARS-CoV-2 kit for BD MAX
AG	BGI, Real-Time Fluorescent RT-PCR Kit for Detecting SARS-CoV-2
AH	BioMerieux Diagnostics, Respiratory panel 2.1 plus
AI	Cepheid, Xpert® Xpress CoV-2/Flu/RSV plus
AJ	Cepheid, Xpert® Xpress SARS-CoV-2
AK	Cepheid, Xpert® Xpress SARS-CoV-2/Flu/RSV
AL	Certest, VIASURE SARS-CoV-2 Real Time PCR
AM	E-gene SARS-CoV-2 specific (unknown); N-gene (unknown)
AN	E-gene SARS-CoV-2 specific (unknown); N-gene, Lu et al. 2020
AO	ELITech Group, SARS-CoV-2 ELITe MGB kit
AP	Eurofins, ViroBOAR 4.0 RT-PCR Kit (SARS-CoV-2)
AQ	GenMark Diagnostics, ePlex® RP2
AR	Gerbion, virellaSARS-CoV-2 seqc REAL TIME RT-PCR KIT
AS	Gold Standard Diagnostics, NovaPrime SARS-CoV-2 (COVID-19)
AT	Gold Standard Diagnostics, NovaPrime TSP SARS-CoV-2 RT-PCR
AU	Hologic, Aptima® SARS-CoV-2 Assay
AV	Hologic, Aptima® SARS-CoV-2/FLU assay
AW	Hologic, Panther Fusion® SARS-CoV-2 assay
AX	N1-gene, Lu et al. 2020; ORF1a/b, CDC China
AY	Pathofinder, RealAccurate® Quadruplex SARS-CoV-2 PCR Kit
AZ	PerkinElmer, SARS-CoV-2 Real-time RT-PCR Assay
BA	PhoenixDx, PDX COVID-19
BB	Procomcure Biotech, PhoenixDx® SARS-CoV-2 Multiplex V2 IVD
BC	Qiagen, NeuMoDx™ Flu A-B/RSV/SARS-CoV-2 Vantage Test
BD	Qiagen, SARS-CoV-2 N1+N2 Assay Kit
BE	r-Biopharm, RIDAGENE SARS-CoV-2 RUO
BF	RdRP-gene, Corman et al. 2020 (adapted)
BG	Roche, Cobas® Liat SARS-CoV-2
BH	Roche, Cobas® Liat SARS-CoV-2/FluA/FluB
BI	Roche, Cobas® SARS-CoV-2: Qualitative assay for use on the Cobas 6800/8800 Systems
BJ	Sarbeco E-gene (unknown)
BK	Sarbeco E-gene (unknown); N-gene (unknown)
BL	Sarbeco E-gene (unknown); N-gene, Lu et al. 2020
BM	Sarbeco E-gene, Corman et al. 2020
BN	Sarbeco E-gene, Corman et al. 2020; N1-gene, Lu et al. 2020
BO	Sarbeco E-gene, Corman et al. 2020; N2-gene, Lu et al. 2020
BP	Sarbeco E-gene, Corman et al. 2020; N-gene, Lu et al. 2020
BQ	Sarbeco E-gene, Corman et al. 2020; RdRP-gene, Corman et al. 2020
BR	Sarbeco E-gene, Corman et al. 2020; RdRP-gene, Corman et al. 2020 (adapted)
BS	Seegene, Allplex™ SARS-CoV-2 Assay
BT	Siemens, FTD Sars-CoV-2 assay
BU	SpeedX, PlexPCR kit
BV	Thermofisher, TaqPath™ COVID-19 CE-IVD RT-PCR
BW	TIB-MolBiol, LightMix® Modular Sarbecovirus E-gene, CE
BX	Xiamen Zeesan Biotech Co., Ltd., SARS-CoV-2 Test Kit (Real-time PCR)

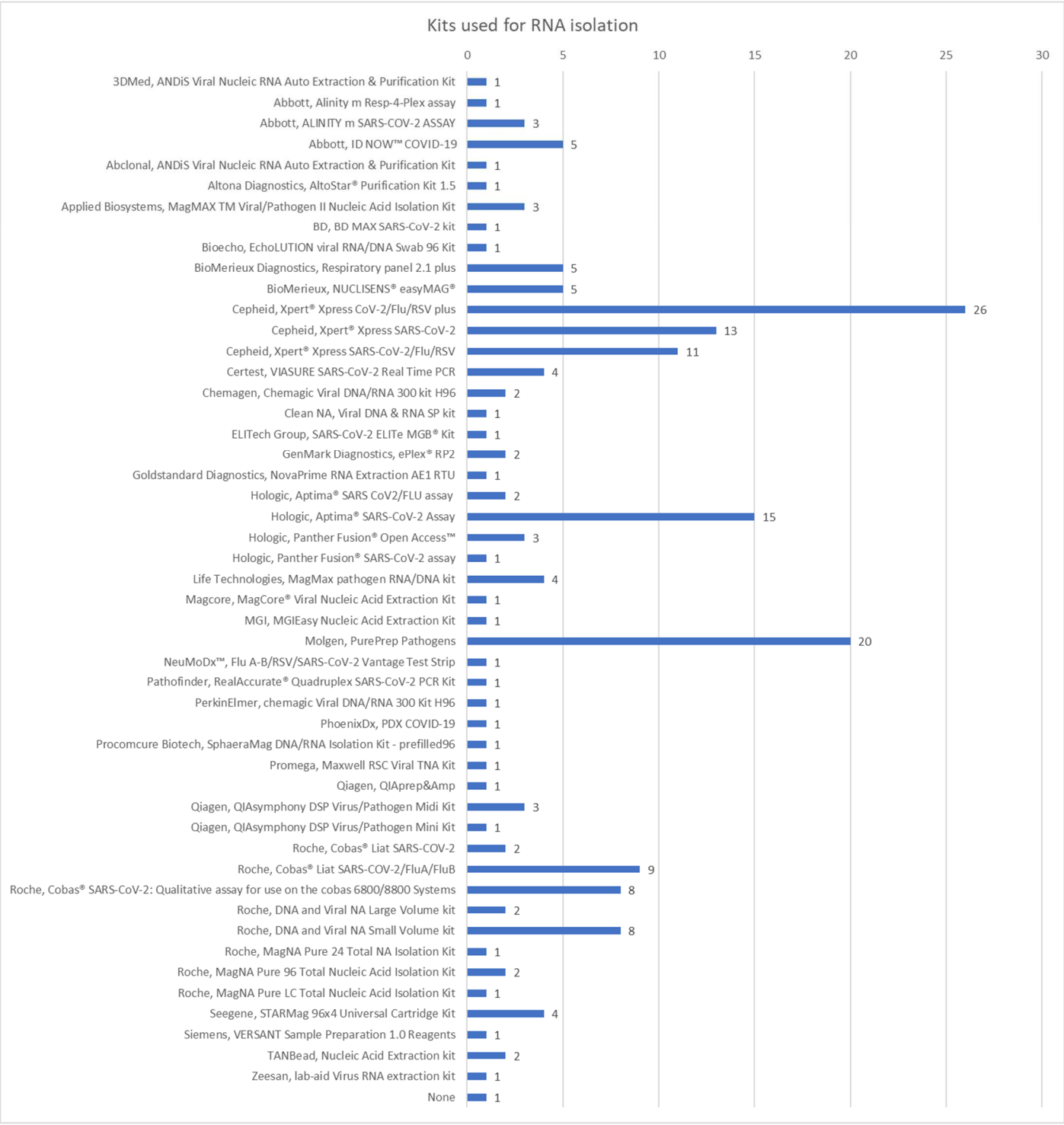
Supplemental figure 4 continued: Legend

6.4 Used equipment, kits, reagents and target genes

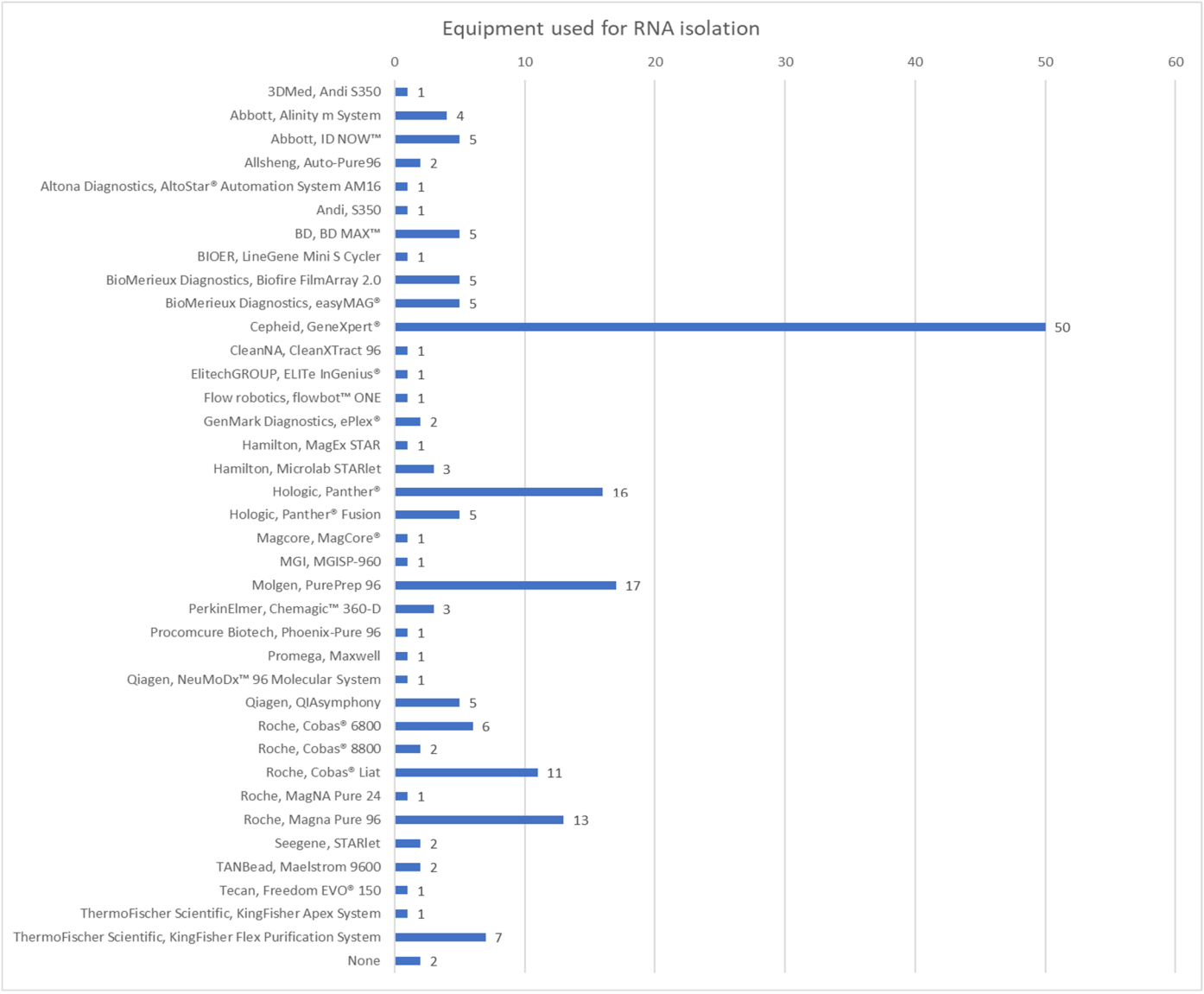
Some of the factors that may determine the performance of the workflows are the kits, equipment and/or separate enzymes and target gene(s) used for extraction and amplification implemented in SARS-CoV-2 diagnostics. Therefore for each workflow these details were inventoried. Combinations of genes used by number of workflows are listed in Supplemental Table 1. Supplemental Figure 5 shows the kits used for RNA/total NA isolation, Supplemental Figure 6 shows the RNA isolation equipment, Supplemental Figure 7 shows the kits used for the RT-PCR or other NAAT reaction, Supplemental Figure 8 shows the separate enzymes used for the in-house RT-PCR or other NAAT reaction and Supplemental Figure 9 shows the equipment used for the RT-PCR or other NAAT reaction. In several occasions the kit used for extraction and for RT-qPCR or other NAAT has the same name because these are all-in-one kits.

Supplemental Table 1. Overview of number and type of target genes used per reported workflow.

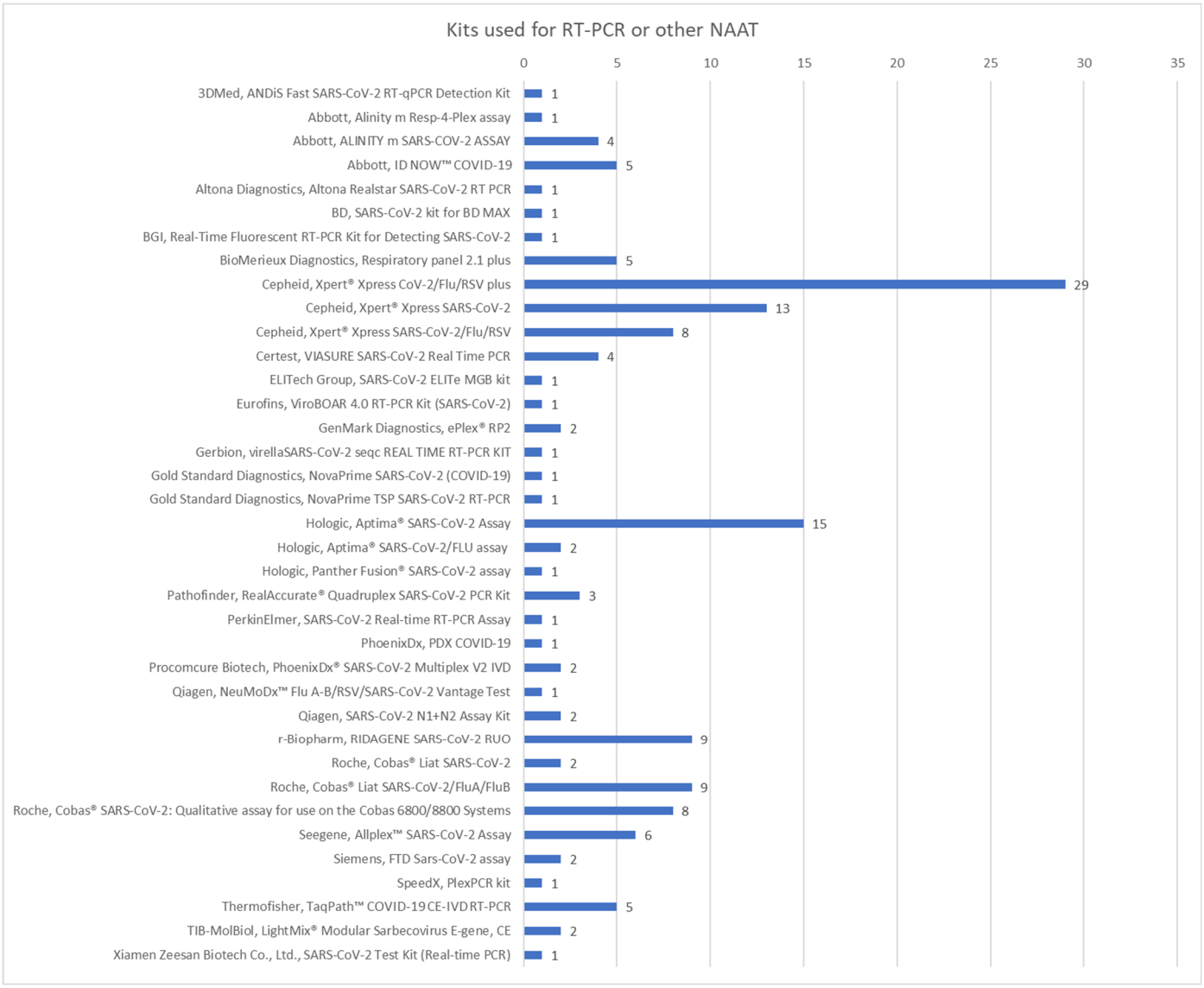
№ target genes in workflow	Target gene(s)	№ workflows
1	E-gene Sarbeco specific	18
	E-gene SARS-CoV-2 specific	10
	N-gene	4
	NSP-2	1
	ORF1a/b	16
	RdRP-gene	6
2	E-gene Sarbeco specific; N-gene	6
	E-gene Sarbeco specific; N1-gene	5
	E-gene Sarbeco specific; N2-gene	27
	E-gene Sarbeco specific; ORF1a/b	7
	E-gene Sarbeco specific; RdRP-gene	3
	E-gene Sarbeco specific; S-gene	1
	E-gene SARS-CoV-2 specific; N-gene	2
	E-gene SARS-CoV-2 specific; RdRP-gene	1
	M-gene; S-gene	5
	N-gene; ORF1a/b	18
	N-gene; RdRP-gene	8
	N1-gene; N2-gene	9
	N1-gene; ORF1a/b	1
	ORF1a/b; ORF1a/b	2
	ORF1a/b; RdRP-gene	1
	ORF8; RdRP-gene	1
3	E-gene Sarbeco specific; N-gene; RdRP-gene	26
	E-gene SARS-CoV-2 specific; N-gene; RdRP-gene	1
	E-gene SARS-CoV-2 specific; N2-gene; RdRP	1
	N-gene; ORF1a/b; S-gene	4
4	E-gene Sarbeco specific; N-gene; RdRP-gene; S-gene	3
	E-gene SARS-CoV-2 specific; N-gene; RdRP-gene; S-gene	1



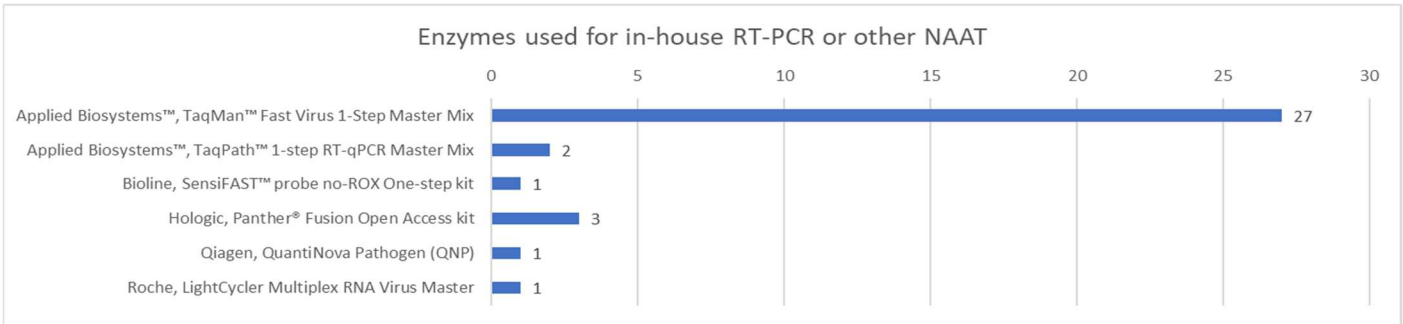
Supplemental Figure 5: The RNA isolation kits used by workflows testing for SARS-CoV-2 together with the number of workflows per kit (n=188)



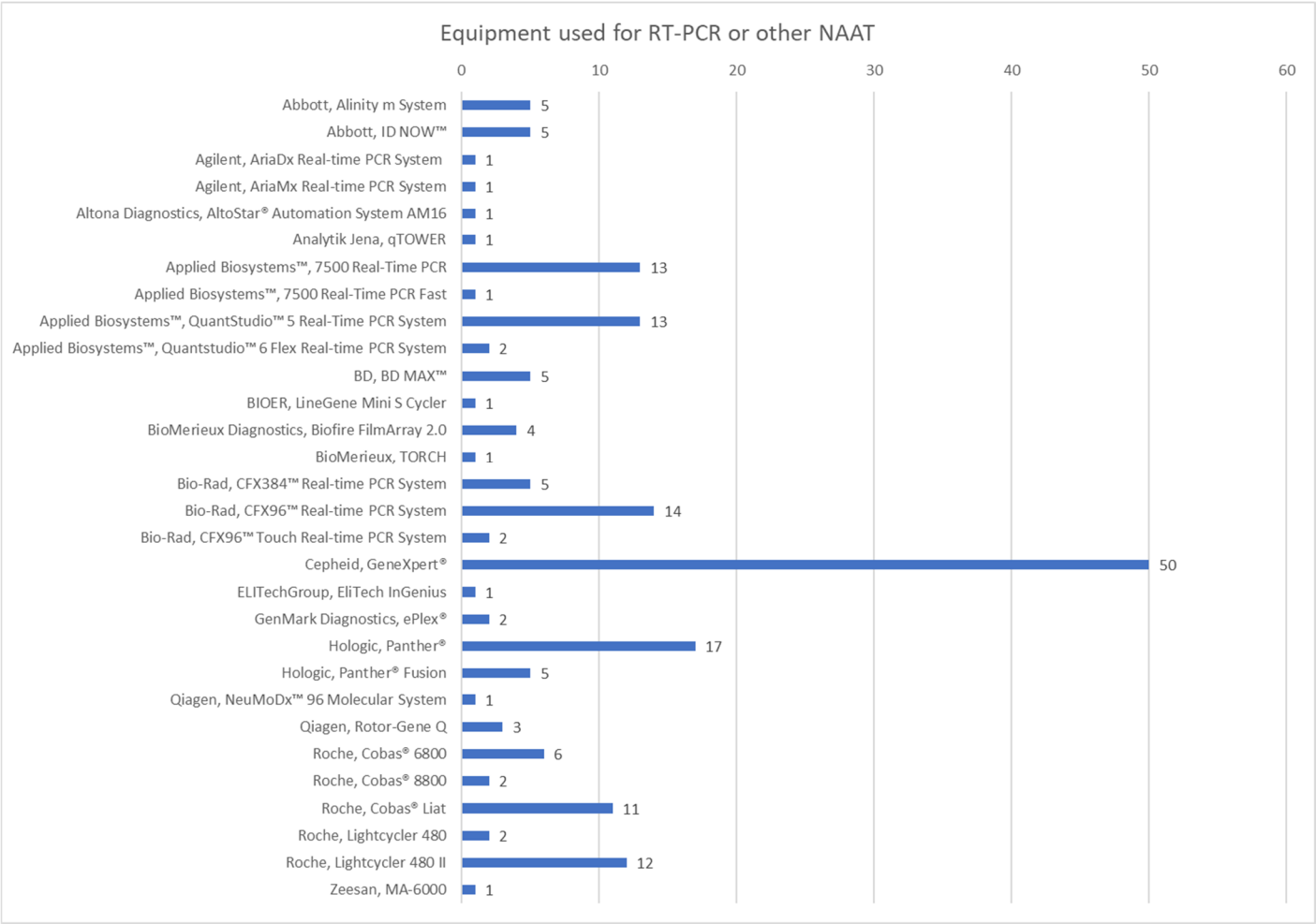
Supplemental Figure 6: The RNA isolation equipment used by workflows testing for SARS-CoV-2 together with the number of workflows per machine (n=188)



Supplemental Figure 7: The RT-qPCR or other NAAT kits used by workflows testing for SARS-CoV-2 together with the number of workflows per kit (n=153). Not all workflows use 'ready to use' kits for their RT-qPCR or other NAAT, so the total N is not equal to the number of workflows tested. For each kit the used target genes are listed. Workflows using separate enzymes and primers and probe are listed in Supplemental Figure 8.



Supplemental Figure 8: The enzymes used for performing RT-PCR or other NAAT by workflows testing for SARS-CoV-2 together with the number of workflows per enzyme (n=35). Not all workflows use separate enzymes for their RT-PCR or other NAAT, so the total n is not equal to the number of workflows tested. Workflows using complete 'ready to use' kits for SARS-CoV-2 detection are listed in Supplemental Figure 7.



Supplemental Figure 9: The RT-PCR or other NAAT equipment used by workflows testing for SARS-CoV-2 together with the number of workflows per machine (n=188)

6.5 Participating laboratories

All participating laboratories are listed below. We would like to thank colleagues from these laboratories for their participation in this round of LEQA for the Dutch SARS-CoV-2 diagnostics field.

Laboratory name
1st lab consortium, Merieux Nutrisciences
1st lab consortium, Nofalab
1st lab consortium, Normec Biobeheer
1st lab consortium, NutriControl B.V.
1st lab consortium, Nutrilab B.V.
1st lab consortium, Triskelion
ADRZ
AmsterdamUMC locatie AMC
ArminLabs
Atalmedial, Medisch Microbiologisch Laboratorium
Brightlabs B.V.
Catharina Ziekenhuis Eindhoven
CBSL Tergooi MC
Certe MMB Friesland en Noordoostpolder
Certe, Medische Diagnostiek en Advies
Comicro B.V.
CWZ medische microbiologie
Deventer Ziekenhuis
Diagnostiek voor U
Diakonessenhuis Utrecht
Erasmus MC, dept Viroscience
Eurofins Clinical Diagnostics - HVL
Eurofins Clinical Diagnostics - NMDL
Eurofins Genomics Europe Applied Genomics GmbH
Eurofins Medische Microbiologie / Alrijne Zorggroep
Franciscus Gasthuis & Vlietland, Medische Microbiologie
Gelre Ziekenhuizen Apeldoorn
GGD Amsterdam
Groene hart Ziekenhuis, afdeling Medische Microbiologie
Haaglanden Medisch Centrum
Hagaziekenhuis, medische microbiologie
HowAreYou Diagnostics
IJssellandziekenhuis, Capelle aan den IJssel
inBiome
Isala Klinieken, LMMI
Jeroen Bosch Ziekenhuis, Regionaal Laboratorium voor Medische Microbiologie en Infectiepreventie
LabMicTA
Labor Dr. Wisplinghoff
Laurentius Ziekenhuis
LHM Diagnostiek
LUMC, KML
Maasstadziekenhuis
MeanderMC
Microbe&Lab B.V

Laboratory name
Microvida
Microvida ETZ
Mozand B.V.
MUMC+ MMB
Noordwest Ziekenhuisgroep Alkmaar
OLVG Lab BV
Pure Medical
Radboudumc
Reinier Haga MDC
Rijnstate Velp, Laboratorium voor Medische Microbiologie en Immunologie
RLM Dordrecht-Gorinchem
Saltro, locatie Hudsondreef
Saltro, locatie Mississippidreef
Sanquin NSS
SKB Winterswijk, KCHL
Slingeland Ziekenhuis Doetinchem, MML
St. Antonius ziekenhuis Nieuwegein
St. Jansdal Ziekenhuis, Medische Microbiologie
Star-shl
Stichting PAMM
Streeklab Haarlem
SYNLAB Belgium sc/SPRL
Synlab Jena Oncoscreen
SYNLAB Leverkusen GmbH
SYNLAB Liège
TLR International Laboratories
UMCG
UMCU, MMB afdeling Klinische Virologie
VieCuri Medisch Centrum Venlo
Wageningen Bioveterinary Research
Ziekenhuis Gelderse Vallei, Medische Microbiologie
Ziekenhuis Rivierenland Tiel, Laboratorium voor medische diagnostiek
Zuyderland MC, Medische Microbiologie