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Automated wash and reuse of disposable pipette tips in a SARS-CoV-2 RT-qPCR diagnostic pipeline

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ABSTRACT

The Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic led to global shortages in laboratory consumables, in particular for automated PCR. The Technical University of Denmark supported Danish hospitals from 2020 to 2022, conducting SARS-CoV-2 RT-qPCR on around 10,000 patient samples daily. We encountered shortages of disposable pipette tips used with automated liquid handlers that transferred oropharyngeal swab samples to 96-well microplates before RNA extraction. To enable tip reuse, we developed an automated protocol for washing tips with a 0.5 % sodium hypochlorite solution. This effectively eliminated carry-over of genomic material and the wash solution remained effective when stored in an open reservoir at ambient temperatures for 24 h. A three-day validation setup demonstrated the robustness of the tip wash protocol. Reducing the number of tips used for transferring samples to 96-well microplates from 96 to 8 enabled us to mitigate pipette tip shortages, lower costs, and minimize plastic waste generation.

1. Introduction

In early 2020, the global dissemination of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) prompted Danish authorities to implement efficient testing strategies, ensuring the swift isolation of infected individuals and their contacts to curb viral spread [1]. As testing capacities were implemented worldwide, the demand for laboratory supplies and consumables for PCR testing increased dramatically, leading to delivery shortages. This impeded large-scale PCR testing in many facilities including our own laboratory at the Centre for Diagnostics, Technical University of Denmark (DTU). We encountered a scarcity of disposable pipette tips suitable for use with the Biomek i5 automated liquid handlers (Beckman Coulter, USA), which were employed for transferring oropharyngeal swab samples from tubes to 96-well plates in a high-throughput SARS-CoV-2 real-time polymerase chain reaction (RT-qPCR) pipeline.

The decontamination and reuse of plasticware, such as disposable pipette tips, is a way to counteract supply shortages, reduce laboratory expenses, and minimize plastic waste. A highly efficient cleaning protocol for pipette tips intended for reuse is important, especially when handling samples for downstream detection of genomic material using the sensitive PCR method. Various cleaning methods for pipette tips have been previously documented. A Decon90™ (potassium hydroxide) method, utilizing a separate device for cleaning disposable pipette tips, has been applied in an immunoassay setup, but its effectiveness in PCR-based setups remains untested [2]. Another method, based on cold plasma technology, is commercially available either as a dedicated cleaning robot or as components that can be integrated into a variety of pipetting robots (IonField Systems, Wilmington, USA). This system efficiently removes genomic material from used disposable pipette tips prior to PCR analysis [3,4]. Furthermore, effective automated sodium hypochlorite wash protocols have been published, describing the decontamination of non-disposable tips, both for the removal of serum components and genomic material [5,6].

Here, we present an effective, automated, cost-effective and easily implementable 0.5 % sodium hypochlorite wash protocol for the decontamination and reuse of disposable pipette tips. This protocol was set up and validated in June 2021, a period during the pandemic marked by the challenges of acquiring laboratory plasticware. It was developed to function as an integrated part of the robotic transfer of oropharyngeal swab samples to 96-well plates within a high-throughput SARS-CoV-2 RT-qPCR pipeline.

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2. Materials and methods

2.1. SARS-CoV-2 RT-qPCR pipeline setup

A SARS-CoV-2 RT-qPCR diagnostic pipeline, consisting of six workstations, was established at DTU (Fig. 1). Briefly, at Station 1, patient oropharyngeal swab samples were registered, placed in 24-well sample racks (4 rows x 6 columns), centrifuged at 2000 RPM for 30 s, and transferred to 96-well microplates (8 rows x 12 columns) (#AB1127, Thermo Scientific) using a Biomek i5 automated liquid handler (Beckman Coulter, USA). At Station 2, viral RNA, from patient samples and from negative patient sample pools spiked with Twist control RNA, was extracted with an RNAdvance Viral Reagent Kit (#C57956, Beckman Coulter, USA) using a Biomek i7 automated workstation (Beckman Coulter, USA). PCR reactions were transferred to Rotor-Discs with 100 wells (#981311, QIAGEN, Germany) at Station 3 using QiAgility (QIAGEN, Germany), and run on QIAGEN Rotor-Gene Qs (QIAGEN, Germany) at Station 4. SARS-CoV-2 negative and positive samples were identified during data analysis at Station 5, and positive samples were sequenced for variant identification at Station 6.

2.2. SARS-CoV-2 positive and negative patient swab samples

Patient oropharyngeal swab samples were delivered from the hospitals in a buffer containing 4M guanidine thiocyanate that lyses virus particles and inhibits enzyme degradation of RNA and DNA. The samples were stored for up to one month before retesting them with the tip wash protocol in a pilot study and a validation study. Negative patient samples were stored at 4 °C, while positive samples were collected and stored at -20 °C as part of the routine shipment of positive samples to hospitals for subsequent storage or analysis.

2.3. SARS-CoV-2 positive controls

Each 96-well plate contained two SARS-CoV-2 positive controls, with either a low concentration (POS LOW, two copies/µL) or a high concentration (POS HIGH, 100 copies/µL) of synthetic RNA (#102019, Twist Bioscience). The synthetic RNA was derived from two loci in the viral nucleocapsid (N) gene (N1 and N2). Oropharyngeal swab samples that had previously tested negative were pooled and used as a matrix for generating spiked positive controls.

2.4. SARS-CoV-2 RT-qPCR

The PCR master mix included primers targeting the two loci on N1 and N2 RNA and primers for human RNase P (RP) DNA, which was used as a swab sampling control [7]. For each PCR reaction, the master mix was prepared, containing 10 µL One Step PrimeScript III RT-qPCR mix (#RR61HW, Takara, Japan) and five µL Covid-19 RT-qPCR Multiplex Assay primer mix (CoviDetect™ #8002, Pentabase, Denmark). Five µL of template was added to the master mix, and RT-qPCR was performed using a two-step program in a Rotor-Gene Q real-time PCR instrument (Qiagen, Germany), according to the PCR kit suppliers' protocol (Table 1).

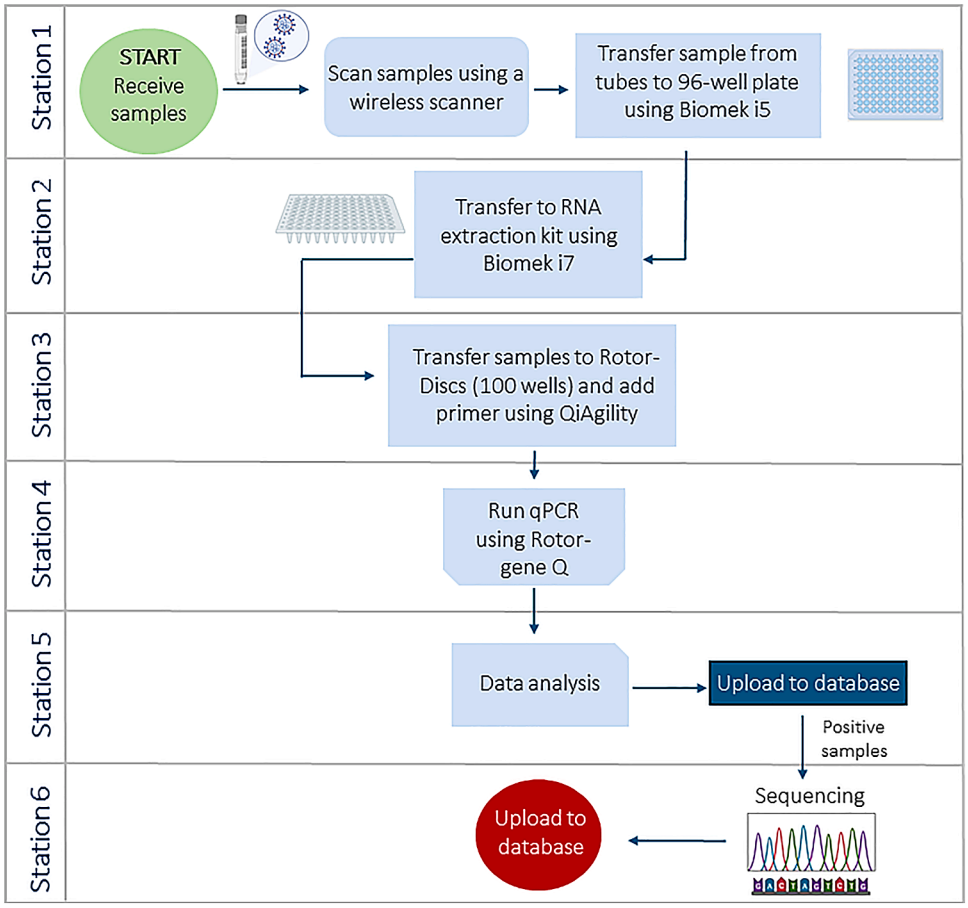

Fig. 1. Flowchart of the SARS-CoV-2 RT-qPCR diagnostic pipeline. The pipeline consists of 6 stations. At Station 1, samples were received, scanned and transferred to 96-well microplates using the Biomek i5 liquid handler, with or without the integrated tip wash protocol. At Station 2, samples were transferred to plates containing an RNA extraction kit and processed using a Biomek i7 liquid handler. At Station 3, samples were transferred to Rotor-Discs and incubated with primers using a QiAgility robot, and RT-qPCR was performed at Station 4 using Rotorgene Qs. Data analysis was conducted at Station 5, and the results were uploaded to a hospital database. At Station 6, positive samples underwent sequencing for variant identification, and these results were also uploaded to the database.

Table 1
RT-qPCR cycling program used with the CoviDetect™ Kit. * Green for N1: FAM/SYBR, yellow for N2: HEX/VIC, red for RP: Cy5.

No.	Step	Temperature (°C)	Time (Sec)	Cycles	Acquisition channels
1	Reverse transcription	52	300	1	
2	PCR activation	95	10	1	
3	Denaturation	95	5	7	
4	Annealing	66	30		
5	Denaturation	95	5		
6	Annealing	60	30	38	Green, yellow, red*

2.5. Tip wash protocol for the Biomek i5 automated liquid handler

Local exhaust ventilation systems, with a suction strength of 250 m^3/hour, were implemented for the Biomek i5 automated liquid handlers to remove potentially toxic fumes resulting from interactions between guanidine thiocyanate-containing lysis buffer and sodium hypochlorite.

The Biomek i5 automated liquid handler allows for the setup of user-specific programs utilizing up to 25 deck positions (Fig. 2). Two positions held an integrated waste container, while six positions were designated for the standard protocol. This left several vacant deck positions for integrating the wash protocol for disposable pipette tips. The wash buffer contained sodium hypochlorite (#425044, 10-15 % active chlorine, Honeywell, USA), diluted in Milli-Q water to a final concentration of 0.5 %.

The reservoirs used for Milli-Q water and the wash buffer were 3D-printed, since the dimensions of commercially available reservoirs did not permit full immersion of the tips during the wash protocol. Their design followed the standard SBS titer-plate format (85.48 mm wide x 127.76 mm long). The required height of the 3D-printed reservoir depends on the type of disposable tip used and the volume required for immersing the part of the tip previously inside the sample tube. Our 3D-printed reservoirs, with a height of 81 mm, could contain a volume of approximately 800 mL.

The Biomek i5 was loaded with a box of disposable 1025 µL pipette tips (#C41862, Beckman Coulter, USA), two 3D-printed reservoirs containing Milli-Q water, a 3D-printed reservoir containing 0.5 % sodium hypochlorite, a rack holding tubes with patient and control samples, a 96-well microplate (#AB1127, Thermo Scientific, USA) and two waste reservoirs (#201244-100, Agilent, USA). The span-8 pod on the Biomek i5 was programmed to pick up eight tips, aspirate 200 µL aliquots from each of the eight samples positioned in columns one and two of the 24-well sample rack, and dispense the entire volume into positions A1 to H1 in the 96-well microplate (steps 1-3, Fig. 2).

Instead of discarding the pipette tips prior to transferring the next eight samples, the tips underwent a wash cycle. First, to remove residual lysis buffer derived from the samples, the tips aspirated 300 µL Milli-Q water from a water reservoir, which was then discarded into a waste reservoir (steps 4 & 5, Fig. 2). The tips then moved to a reservoir containing 300 mL of 0.5 % sodium hypochlorite wash buffer (step 6, Fig. 2), aspirating and dispensing 500 µL wash buffer in five cycles over 15 s. To prevent sample-to-sample carryover, the part of the tip that had been inside the sample tube was fully immersed. Finally, to remove any remaining wash buffer, the tips aspirated 600 µL Milli-Q water from a second water reservoir (step 7, Fig. 2), discarding it into a new waste reservoir (Step 8, Fig. 2). This protocol was repeated until all samples had been transferred from the four 24-well sample racks to the 96-well microplate. The described workflow, along with the positions of tips, reservoirs, sample racks and 96-well microplate, was programmed into the Biomek i5 software (Biomek i5 Software version 5.0).

2.6. Pilot study design

A pilot study was conducted to assess either potential RNA carry-over or loss of sensitivity due to sample degradation when reusing washed tips six times to transfer SARS-CoV-2 positive and negative samples. Additionally, the study aimed to evaluate the stability and efficacy of the 0.5 % sodium hypochlorite wash buffer.

In the pilot study, the same eight tips were used to sequentially transfer a dilution series of synthetic SARS-CoV-2 Twist RNA and guanidine thiocyanate lysis buffer (4M) to the first six columns of a 96-well microplate using intermittent wash steps. This was done to test if the wash buffer reliably removes contamination when reusing washed tips to transfer samples with RNA concentrations ranging from 0.05 to 500,000 copies/µL (Table 2). A wider range of concentrations between 0.05 and 0.5 copies were tested in parallel without the wash steps to determine the limit of detection (LOD) of the N1 and N2 assays (Table 3).

The pilot study also included the testing of a panel of positive samples, consisting of seven human oropharyngeal swab samples that had been stored at -20°C and were previously tested positive for SARS-CoV-2 in our pipeline, exhibiting a range of low to high Ct values. The positive extraction control containing Twist synthetic SARS-CoV-2 RNA was also

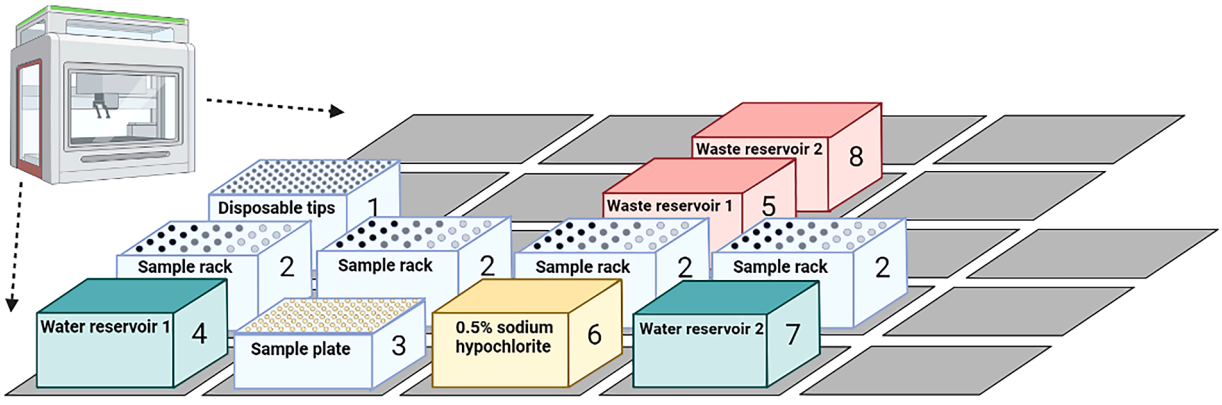

Fig. 2. Automated tip wash protocol setup on the Biomek i5 automated liquid handler deck. The Biomek i5 features a span-8 pod for disposable tips capable of handling eight individual samples simultaneously. The deck has 25 positions of which two are reserved for a waste container, and it therefore offers 23 free deck positions onto which plasticware, samples, control samples, and buffers can be loaded. As indicated, the protocol comprises eight sequential steps: loading eight disposable tips onto the span-8 pod (step 1), transferring eight sample aliquots from a sample rack (step 2) to a 96-well microplate (step 3), washing the tips in a water reservoir (step 4), transferring waste to a waste reservoir (step 5), washing the tips with 0.5 % sodium hypochlorite (step 6), performing a subsequent water rinse (step 7), and transferring waste to a waste reservoir (step 8). The tip wash protocol is repeated until all samples from the sample racks are transferred. The figure was created using Biorender.com.

Table 2
Ct values for N1 and N2 when reusing washed pipette tips for sequential addition of a dilution series of synthetic SARS-CoV-2 Twist RNA, or lysis buffer (LB) to the first six columns of a 96-well microplate. The same eight pipette tips were used six times in each plate row with intermittent wash steps. ND = not detected/negative.

Plate column	1		2		3		4		5		6			
Sample type	Twist RNA		LB		Twist RNA		LB		Twist RNA		LB			
Twist SARS-CoV-2 RNA concentration (copies/μL) Nucleocapsid (N) gene	N1 Ct	N2 Ct	N1 Ct	N2 Ct	N1 Ct	N2 Ct	N1 Ct	N2 Ct	N1 Ct	N2 Ct	N1 Ct	N2 Ct	N1 Mean Ct	N2 Mean Ct
500,000	8.4	8.2	ND	ND	8.1	7.9	ND	ND	8.3	8.1	ND	ND	8.3	8.1
50,000	12.1	11.7	ND	ND	11.8	11.5	ND	ND	12.2	11.6	ND	ND	12.0	11.6
5,000	15.8	15.2	ND	ND	15.6	15.1	ND	ND	15.7	15.0	ND	ND	15.7	15.1
500	18.8	18.4	ND	ND	19.0	18.4	ND	ND	19.1	18.3	ND	ND	19.0	18.4
50	22.2	21.4	ND	ND	22.1	21.4	ND	ND	21.8	21.3	ND	ND	22.0	21.4
5.0	25.3	24.7	ND	ND	25.6	25.0	ND	ND	26.4	24.7	ND	ND	25.8	24.8
0.5	29.8	29.4	ND	ND	29.0	28.6	ND	ND	28.8	28.5	ND	ND	29.2	28.9
0.05	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 3
Dilution series of synthetic SARS-CoV-2 Twist RNA showing the measured Ct values in relation to RNA concentration and the limits of detection for the N1 and the N2 assays. Ct values are shown in triplicate together with the average Ct value for each RNA concentration (Rep Ct).

Twist SARS-CoV-2 RNA concentration		N1 (Ct values)				N2 (Ct values)			
Copies/μL	Copies/sample (5 μL)	1	2	3	Rep Ct	1	2	3	Rep Ct
500,000	2,500,000	8.4	8.1	8.3	8.3	8.2	7.9	8.1	8.1
50,000	250,000	12.1	11.8	12.2	12.0	11.7	11.5	11.6	11.6
5,000	25,000	15.8	15.6	15.7	15.7	15.2	15.1	15.0	15.1
500	2,500	18.8	19.0	19.1	19.0	18.4	18.4	18.3	18.4
50	250	22.2	22.1	21.8	22.0	21.4	21.4	21.3	21.4
5	25	25.3	25.6	26.4	25.8	24.7	25.0	24.7	24.8
0.5	2.5	29.8	29.0	28.8	29.2	29.4	28.6	28.5	28.9
0.4	2	29.57	30.26	30.16	30.0	28.7	28.1	28.1	28.28
0.2	1	31.17	29.26	ND	30.2	27.85	28.75	28.21	28.27
0.1	0.5	ND	ND	ND	ND	33.64	ND	ND	ND
0.05	0.25	ND	ND	ND	ND	ND	ND	ND	ND

included (POS HIGH, 100 copies/μL (Table 4). A guanidine thiocyanate lysis buffer (4M) was added to eight tubes serving as negative samples to assess potential RNA carry-over due to tip reuse.

The positive samples were placed in the first two columns, while the tubes containing lysis buffer were added to the third and fourth column of a 24-well sample tube rack. Using the same eight pipette tips, the Biomek i5 tip wash protocol sequentially transferred positive samples and lysis buffer to the first six columns of a 96-well microplate, with or without intermittent wash steps. The final positioning of samples and controls on the microplate is shown in Table 5.

First, aliquots from the eight tubes with positive samples were transferred to column one on the 96-well microplate. The tips were then washed and reused to transfer lysis buffer samples to column two (Wash 1). The process was repeated, with the tips used again to transfer new aliquots from the positive sample tubes to column three (Wash 2). In the next step, the tips were reused without washing (No wash) for transferring the lysis buffer to column four. To assess the potential negative impact of the wash buffer carry-over between samples (reflected in higher Ct values), the wash step was repeated, and positive sample aliquots were added to columns five and six with an additional intermittent wash step (Wash 3-4).

Table 4
Original N1, N2 and RP Ct values for the sample panel used in the pilot study. The panel included seven SARS-CoV-2 positive swab samples (S1 to S7) and the positive extraction control (POS HIGH) containing 100 copies/sample of Twist SARS-CoV-2 RNA.

Sample ID	N1 (Ct values)	N2 (Ct values)	RP (Ct values)
S1	10.4	10.1	21.9
S2	14.0	13.8	22.0
S3	14.2	14.0	23.4
S4	18.7	18.5	20.9
S5	22.5	22.4	30.1
S6	25.1	24.2	22.6
S7	27.8	27.0	31.8
POS HIGH	23.2	22.7	19.4

Table 5
Final positioning of samples from the pilot study panel in the first six columns of a 96-well microplate. Seven SARS-CoV-2 positive swab samples (S1 to S7) and a positive controls (PC) containing 100 copies/sample of Twist SARS-CoV-2 RNA were positioned in row A-H, columns 1, 3, 5 and 6. Lysis buffer (LB), serving as a negative control to test for RNA-contamination after tip wash, was positioned in row A-H, column 2 and 4. The top row indicates whether a fresh tip was used or if a wash step was performed prior to sample transfer.

Row/column	Fresh tip 1	Wash 1 2	Wash 2 3	No wash 4	Wash 3 5	Wash 4 6
A	S1	LB	S1	LB	S1	S1
B	S2	LB	S2	LB	S2	S2
C	S3	LB	S3	LB	S3	S3
D	S4	LB	S4	LB	S4	S4
E	S5	LB	S5	LB	S5	S5
F	S6	LB	S6	LB	S6	S6
G	S7	LB	S7	LB	S7	S7
H	PC	LB	PC	LB	PC	PC

To assess the stability and efficacy of the 0.5 % sodium hypochlorite wash buffer, the described assay setup was repeated three times using a 0.5 % sodium hypochlorite solution either prepared freshly (0 h) or stored in a reservoir on the Biomek i5 deck for 8 or 24 h at ambient temperature (Table 6). Before each use and at each time point, the pH of the wash buffer was measured using a pH meter (PHM220, Radiometer Copenhagen, Denmark, pH electrode: Sentix 82, WTW, USA).

2.7. Validation study design

A validation study was conducted to evaluate the diagnostic

Table 6
Pilot study results from testing carry-over of N1, N2, and RP genomic material to downstream negative control samples, which consisted of lysis buffer (LB, Wash 1, and No wash), and testing for wash buffer-mediated RNA/DNA degradation (Wash 2, 3 & 4). Ct-values are presented for S1 or S6 and lysis buffer transfer using the same tip (tip 1 or 6) across six robotic steps, with or without a sodium hypochlorite washing step and with varying buffer storage times (0, 8 or 24 h). Ct values for S2-S5 and S7 are shown in supplementary Table 1 (S2-7, Supplementary Table 1). Presented are mean Ct values, standard deviations, and CV percentages for the two positive samples (S1 and S6). ND = not detected/negative.

				Ct-values at varying wash buffer storage time and pH								
				0 h, pH 11.36			8 h, pH 10.33			24 h, pH 9.32		
Tip	Use	Wash	Sample	N1	N2	RP	N1	N2	RP	N1	N2	RP
1	1	Fresh tip	S1	9.4	9.5	21.2	8.9	9.1	20.8	10.1	9.6	22.0
	2	1	LB	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	2	S1	9.3	9.6	20.8	9.4	9.3	20.6	9.3	9.0	20.9
	4	No wash	LB	16.2	16.4	28.2	16.3	16.2	30.5	17.4	17.3	33.4
	5	3	S1	9.6	9.6	21.0	9.3	9.1	20.5	9.3	9.0	20.6
	6	4	S1	9.6	9.8	20.8	9.3	9.3	20.7	9.3	9.1	20.6
Mean Ct value (S1)				9.5	9.6	20.9	9.2	9.2	20.6	9.5	9.2	21.0
Standard deviation (S1)				0.2	0.1	0.2	0.2	0.1	0.1	0.4	0.3	0.7
CV% (S1)				1.6	1.2	0.9	2.3	1.2	0.7	4.4	2.9	3.2
6	1	Fresh tip	S6	23.5	22.8	21.6	23.5	22.9	22.4	24.8	23.6	22.1
	2	1	LB	ND	ND	ND	ND	ND	ND	ND	ND	ND
	3	2	S6	23.4	22.9	21.9	23.8	23.2	21.7	23.6	22.6	21.4
	4	No wash	LB	ND	ND	ND	33.5	ND	ND	ND	ND	ND
	5	3	S6	23.6	23.4	21.5	23.8	22.9	21.8	23.4	23.0	21.6
	6	4	S6	23.2	23.0	21.0	23.4	22.8	21.4	22.8	22.6	21.0
Mean Ct value (S6)				23.4	23.0	21.5	23.7	23.0	21.8	23.7	23.0	21.5
Standard deviation (S6)				0.2	0.2	0.4	0.2	0.2	0.4	0.8	0.5	0.5
CV% (S6)				0.9	1.1	1.7	0.8	0.7	1.8	3.5	2.1	2.1

accuracy of the SARS-CoV-2 RT-qPCR assay when employing the tip wash protocol instead of the original standard protocol for transferring oropharyngeal swab samples to 96-well microplates before RNA extraction.

The tip wash protocol was tested over a period of three days in our high-throughput pipeline using 1672 patient samples that had previously been tested positive (n = 95) or negative (n = 1577) with the standard protocol. The samples were transferred to 19 96-well microplates in the Biomek i5 using the tip wash protocol. The same eight tips were used for transferring samples from plate column 1 to 12 with intermittent wash steps. Eight plates were run on day 1, five plates on day 2, and six plates on day 3. Each 96-well microplate contained seven positive samples and 89 negative samples. The positive samples included five patient samples as well as a POS LOW and a POS HIGH control containing synthetic RNA from the SARS-CoV-2 N1 and N2 gene. The negative samples included 83 patient samples as well as six water controls. To simulate a realistic scenario, positive samples and controls were interspersed randomly among the negative samples.

A volume of 800 mL 0.5 % sodium hypochlorite was added to a reservoir, and 800 mL of Milli-Q water was added to each of the two water reservoirs. The reagents were changed every 24 h.

The results for the patient samples were compared to the results previously obtained when testing the same samples using the standard protocol.

2.8. Data analysis

The diagnostic accuracy of the integrated pipette tip wash protocol was measured by calculating the true positive, false positive, false negative and true negative matrix, as well as percent sensitivity, percent specificity, and kappa values [8]. Only results for patient samples were included in the data analysis, as the POS LOW, POS HIGH, and water samples were used solely as internal assay quality controls on each plate (Table 7).

Patient samples positive for N1 and/or N2 expression as well as RP expression using the standard protocol were categorized as true positives in the data analysis. Conversely, patient samples that were negative for N1 and N2 expression and positive for RP using the standard protocol were categorized as true negatives. Results for samples that tested negative for RP using the standard protocol (n = 22) were excluded from the statistical analysis due to ambiguity over true negative or positive status. Among these samples, four samples were positive for N1 and/or

Table 7
2 × 2 contingency tables with results from the final validation of the tip wash protocol compared to the standard protocol without an integrated wash protocol. The table shows counts of true positives, false positives, false negatives and true negatives, percent diagnostic sensitivity and specificity, and the kappa value for A) N1 expression, B) N2 expression, and C) RP expression. Data analysis included results from a total of 1650 patient samples. NA= Not Applicable

A) Tip wash protocol (N1)	Standard protocol (N1)		
	Positive	Negative	Totals
Positive	90	2	92
Negative	0	1558	1558
Totals	90	1560	1650
Diagnostic sensitivity = 100			
Diagnostic specificity = 99.9			
Kappa value = 1.00			
B Tip wash protocol (N2)	Standard protocol (N2)		
	Positive	Negative	Totals
Positive	84	1	85
Negative	7	1558	1565
Totals	91	1559	1650
Diagnostic sensitivity = 92.3			
Diagnostic specificity = 99.9			
Kappa value = 0.99			
C) Tip wash protocol (RP)	Standard protocol (RP)		
	Positive	Negative	Totals
Positive	1623	0	1623
Negative	27	0	27
Totals	1650	0	1650
Diagnostic sensitivity = 98.4			
Diagnostic specificity = NA			
Kappa value = 0.98			

N2 and 18 samples were negative for N1 and/or N2. Data comparison was performed separately for each of the N1, N2 and RP expression profiles. For example, a patient sample positive only for N1 expression using the standard protocol was considered a true negative for N2.

Since some results were excluded from the data analysis as described above, the data analysis covered 1650 paired results for N1, N2, and human RP gene expression in patient samples, comprising positive (n = 91) and negative (n = 1559) patient samples (Table 7).

3. Results

3.1. Pilot study results

When using the same tips for six sequential transfers of samples from a dilution series of SARS-CoV-2 Twist RNA (0.05 to 500,000 copies/μL) and lysis buffer, no RNA was detectable in the transferred lysis buffer (Table 2). This demonstrates the high efficacy of the tip wash protocol in removing RNA from the tips. The average Ct values of the transferred sample aliquots containing 500,000 copies/μL were N1 = 8.3 and N2 = 8.1. In the standard pipeline, samples with Ct values lower than 10 for N1 and N2 were seldom encountered, as evidenced by the box plots in Fig. 4, which shows the range of Ct values detected in the validation study. The limit of detection of the SARS-CoV-2 PCR assay, when testing a sample volume of five μL, was shown to be between 1 to 2 copies of N1 RNA and 0.5 to 1 copies of N2 RNA (Table 3).

To further evaluate the effectiveness of the tip wash protocol, including integrity of the wash buffer, a small panel of patient samples was tested (Table 4). Since the risk of sample-to-lysis buffer carry-over depends on the Ct value of the positive sample, we provide detailed results for sample one (S1), which has the lowest Ct value. Additionally, we elaborate on sample six (S6), characterized by a high Ct value closer to the assay cut-off, serving as an indicator of potential reduced analytical sensitivity due either to the extra freeze-thaw cycle or sample degradation by the lysis buffer. Although sample seven exhibited the highest Ct values, it was near the assay cut-off, limiting its utility as a clear indicator of sample degradation. Results for the remaining samples (S2-5, S7) are shown in Supplementary Table 1. Furthermore, we only elaborate on the part of the study utilizing freshly prepared sodium hypochlorite solution for the washing steps (0 h, Table 6).

The Ct values for S1, transferred to the 96-well microplate with a fresh tip, were N1 = 9.4, N2 = 9.5 and RP = 21.2 (tip 1, use 1, Table 6). When the tip used for transferring S1 was washed before transferring the lysis buffer to the plate, no Ct signal was detected in the lysis buffer in the downstream PCR analysis (tip 1, use 2, Table 6). Thus, the tip wash protocol eliminated RNA carry-over between the sample and lysis buffer.

Transferring the lysis buffer using the same tip that had been used to transfer S1, without an intermittent wash step, resulted in positive Ct values of N1 = 16.2, N2 = 16.4 and RP = 28.2 (tip 1, use 4, Table 6). This demonstrated RNA carry-over when omitting the tip wash protocol and confirmed the complete removal of genomic material by the wash protocol.

The Ct values for S6, transferred to the 96-well microplate with a fresh tip, were N1 = 23.5, N2 = 22.8 and RP = 21.6 (tip 6, use 1, Table 6). Upon washing and reusing the same tip to transfer S6 to the plate (Wash 2), similar Ct values were measured (N1 = 23.4, N2 = 22.9 and RP = 21.9) (tip 6, use 3, Table 6). This showed that the sodium hypochlorite wash buffer in the tips was adequately removed during the wash step with water and had no adverse impact on the downstream PCR reaction.

To assess any potential effect of accumulated wash buffer, two extra cycles of sample transfer and tip wash protocol were performed using the same tip (tip 6, use 5 & 6). For S6 this yielded Ct values of N1 = 23.6 and 23.2, N2 = 23.4 and 23.0 and RP = 21.5 and 21, mirroring the values obtained with a fresh tip, demonstrating that any accumulated wash buffer did not affect the result.

Transferring S2-5 and S7, using the same procedure as described above, yielded similar results, showing no sample-to-lysis buffer carry-over (Supplementary Table 1). Testing S1 to S7 with the three buffer conditions resulted in three sets of Ct values for N1, N2 and RP, exhibiting acceptable CV percentages (CV% <10) within the range of 0.2 to 8.6 (Table 6, Supplementary Table 1). Thus, the repeated wash steps did not affect the surface property of the plastic tips, which could otherwise diminish handling precision and assay reproducibility.

The pH of the freshly prepared sodium hypochlorite solution was 11.4. After eight hours, the pH dropped to 10.3, and after 24 h, it reached 9.3 (Table 5). Despite the pH decrease, the wash buffer's integrity remained unaffected, as seen by the absence of Ct values at tip use 2 for S1 at all three time points, suggesting it is sufficient to change it once daily (tip use 2, Table 6).

The results from the pilot study indicate that the tip wash protocol effectively eliminates the risk of RNA carry-over when using the same tip six times and that any accumulated lysis buffer in the tip does not affect analytical sensitivity. Since using the tip six times did not result in any RNA carry-over or diminished sensitivity, we proceeded to perform a final validation in our high-throughput setup where each tip was used 12 times.

3.2. Validation study results

Diagnostic sensitivities, specificities, and kappa values were calculated by comparing the results to those originally generated using the standard protocol (without the tip wash) in a true positive, false positive, false negative, and true negative matrix (Table 6).

The kappa values of 0.98 to 1.00 are close to one, which indicates a strong agreement with the standard protocol. The assay setup with the integrated tip wash protocol setup achieved high diagnostic sensitivity (DSe) and specificity (DSp): 100 % DSe and 99.9 % DSp for N1 expression, 92.3 % DSe and 99.9 % DSp for N2 expression, and 98.4 % DSe for RP expression. Two samples exhibited false positive results, either for the N1 gene or for both N1 and N2 genes (Table 7).

As shown in Table 6, seven samples were false negative for N2 and 27 for RP. The false negatives were evenly distributed according to the number of times a tip was reused, indicating that it is neither the wash procedure nor any remaining wash buffer in the tips that caused the false negatives (Fig. 3).

The median Ct values for N1 and N2 exhibited a significant increase when samples were retested using the tip wash protocol (Ct_{N1} = 19.3, Ct_{N2} = 18.3), compared to the standard protocol (Ct_{N1} = 17.7, Ct_{N2} = 16.9). The median RP Ct values were similar when using the tip wash protocol (Ct_{RP} = 19.7) compared to the standard protocol (Ct_{RP} = 19.5). These results indicate that the extra freeze-thaw cycle or sample storage caused slight degradation of the N1 and N2 RNA, but not of RP DNA

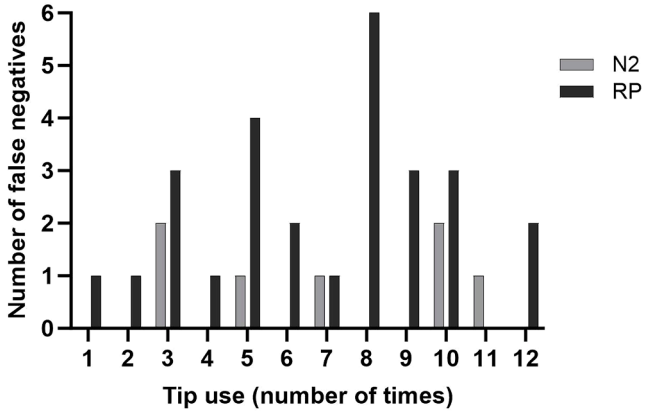

Fig. 3. The distribution of false negative results for N2 or RP expression in relation to the number of times each tip was used in the validation study.

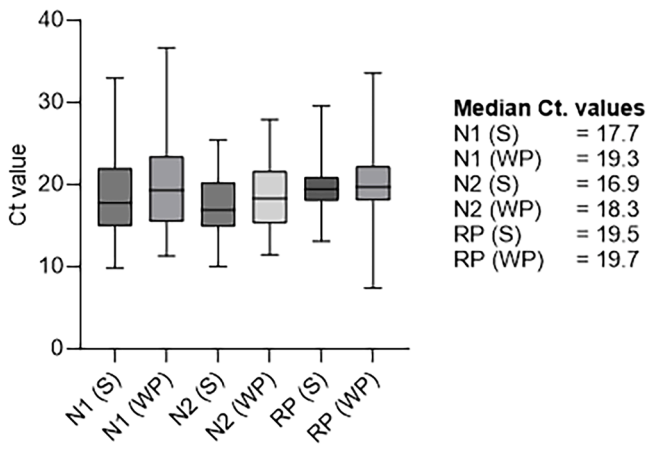

Fig. 4. Box plot showing the range of N1, N2 and RP Ct values measured in the validation study when using either the standard protocol (S) or the tip wash protocol (WP) to transfer samples to 96-well plates in the Biomek i5 liquid handler. The figureonly includes results for samples that were positive for N1, N2 or RP using both the standard and the tip wash protocol.

(Fig. 4).

4. Discussion

The pilot study, utilizing disposable pipette tips washed and used six times, demonstrated the elimination of RNA carry-over between human oropharyngeal swab samples and negative lysis buffer controls through our automated tip wash protocol. No degradation of samples was observed due to any accumulation of sodium hypochlorite in water reservoir 2 or traces of wash buffer in the tips, as indicated by the downstream PCR Ct values, which remained unchanged (Table 6, Supplementary Table 1).

The validation study, conducted over three days, demonstrates the feasibility and robustness of the tip wash protocol for daily routine use (Table 7). Potential sources of false-negative results in the final validation study could stem from two factors: either sample degradation due to accumulated wash buffer in the tips when washing and using each tip 12 times, or from the extra freeze-thaw cycle and storage introduced before testing the samples with the tip wash protocol. The distribution of false negatives across the 96-well plates did not correlate with the number of times a tip was used (Fig. 2), suggesting that sample degradation from accumulated wash buffer in the tips was unlikely. However, the median Ct values for N1 and N2 RNA significantly increased when retesting samples with the tip wash protocol (Fig. 4), suggesting that the additional freeze-thaw cycle or RNAse degradation during sample storage led to slight RNA degradation. Notably, the median Ct values for RP remained consistent when using the tip wash protocol compared to the standard protocol, indicating that the extra freeze-thaw cycle did not affect RP DNA, likely due to its lower susceptibility to degradation than RNA (Fig. 4). Ideally, samples would have been tested in parallel with both the standard protocol and the tip wash protocol at the same time using the same number of freeze-thaw cycles. Although the N2 false negatives may be attributed to the sample storage, the RP false negatives must stem from a lack of sample volume transferred to the 96-well microplates during the tip wash protocol, a recurring issue using the standard protocol as well.

The high specificity of the assay, utilizing the tip wash protocol, demonstrates that using the same volume of wash buffer throughout the day does not present a risk of cross-contamination from washing tips that were used to transfer positive samples with high viral loads. However, if any signs of cross-contamination are observed, the wash buffer can be changed more frequently, and swabbing of the instrument can be performed to test whether cleaning the instrument is necessary.

The false positive samples observed in the validation study were caused by cross-contamination from adjacent positive samples. These specific samples were transferred using either a fresh tip or a washed tip that had only been used to transfer SARS-CoV-2 negative sample material, thus ruling out tip reuse as the cause of contamination. Cross-contamination was also a recurring issue when using the standard protocol. To address this challenge, a monitoring system was implemented. This system was based on an algorithm integrated into an Excel sheet for data analysis, enabling the identification of false positive samples. Samples with Cts at least 5 Cts higher than neighboring samples were flagged, and these samples were subsequently rerun to eliminate false positive results.

The SARS-CoV-2 pipeline operated continuously in three shifts, which made it feasible to produce a fresh batch of sodium hypochlorite wash buffer at the start of the morning shift. This newly prepared buffer could then be left at ambient temperature in an open reservoir on the Biomek i5 deck throughout the day and night. We tested whether the quality of the wash buffer was compromised when stored for up to 24 h under these conditions. The stability of the sodium hypochlorite wash buffer is highly dependent on its pH value. The abundance of hypochlorite anions (OCl^-) increases proportionally with rising pH and predominates at a pH above eight. Conversely, at a pH below eight, undissociated hypochlorite (HOCl) predominates and its abundance increases as pH declines. Despite being a more potent decontaminant than OCl^-, HOCl is highly unstable and quickly loses its detergent effectiveness upon exposure to oxygen and ambient temperatures. Studies indicate that sodium hypochlorite solutions of 0.05 to 0.1 % should ideally maintain a pH of 8 to sustain detergent efficiency [9]. The pH of our wash buffer was suboptimal, starting at 11.36 but decreasing to 9.32 after 24 h in an open container at ambient temperatures. The rapid pH drop was attributed to continuous exposure to oxygen and ambient temperature during storage on the robot deck in the open reservoir. Although the buffer should have a reduced decontamination potency at higher pH levels, it still proved potent enough to effectively eliminate RNA carry-over.

The lysis buffer (containing thiocyanate) as well as sodium hypochlorite, may accumulate in water reservoirs 1 and 2 due to carry-over by the tips. In the event of lysis buffer accumulation in water reservoir 1, it could be dragged to the sodium hypochlorite reservoir, resulting in the release of toxic cyanide gas. Safety risks due to potential production of toxic gasses by the tip wash protocol can be eliminated by installing a proper ventilation system in connection to the liquid handler.

The tip wash protocol requires only a few additional consumables beyond the standard protocol, including five reservoirs of which three were 3D printed in-house, as well as Milli-Q water and a sodium hypochlorite stock solution. Compared to the standard protocol, it increases sample-processing time per 96-well microplate by ten minutes, which would not cause any noteworthy delays in response times for Danish hospitals and patients. It is adaptable to diverse types of automated liquid handlers found in high-end laboratories, eliminating the need to invest in new equipment tailored to this task. However, should the tip wash protocol be used for processing samples to detect analytes other than SARS-CoV-2 or with sample matrices other than human oropharyngeal swab samples, a revalidation is recommended.

The protocol, set up in June 2021 in response to the scarcity of laboratory plasticware, was never implemented in our workflow due to the pandemic fading out, which led to a sharp decline in the number of samples tested in Denmark shortly thereafter. Further experience with the protocol was therefore not gained as part of prospective studies.

The tip wash protocol reduces the number of tips required for transferring samples to a 96-well microplate from 96 tips to just eight, without the need for extra equipment and without any interruption to the high-throughput workflow routine. With our daily processing average of approximately 104 96-well microplates, implementing this adjustment would have reduced tip usage by 9153 tips per day. This reduction would have allowed DTU to maintain the pipeline's operation

during extended periods of robotic plasticware shortage.

Our sodium hypochlorite tip wash protocol for Biomek i5 automated liquid handlers is effective and robust, facilitating the reuse of high-demand disposable tips within an automated, high-throughput SARS-CoV-2 RT-qPCR diagnostic pipeline. A tip wash protocol similar to ours has not been documented, as previously published tip wash protocols either require a separate cleaning device or have only been documented to work with non-disposable tips [2–6]. We believe that the protocol is applicable beyond times of supply shortages, as reusing disposable pipette tips is cost-effective due to their relatively high expense and contributes to sustainability by reducing environmental waste.

Ethical statement

Testing human samples in our routine diagnostics pipeline did not require approval by an ethics committee since the work was performed on a contractual basis, the sample IDs were blinded and the residual material had no further use in our facilities.

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CRediT authorship contribution statement

Sanne S. Berger: Validation, Formal analysis, Writing – review & editing. **Jonas Høgberg:** Conceptualization, Investigation, Methodology, Writing – original draft. **Franziska Kuntke:** Conceptualization, Investigation, Methodology, Writing – original draft. **Louisa Obari:** Methodology, Writing – review & editing. **Helene Larsen:** Conceptualization, Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.diagmicrobio.2024.116241.

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