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Research Article

Evaluation and Validation of the AusDiagnostics MT-Prep XL and AusDiagnostics Ultraplex-3 [SARS-CoV-2, Influenza and RSV 8-well Assay]

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Abstract

A validation study was conducted on the AusDiagnostics MT-Prep XL and AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay performed on the AusDiagnostics Ultraplex 3 system. The Cycle threshold (Ct) of clinical patient samples spiked with a positive control, was compared to the reference laboratory standard, AusDiagnostics MT-Prep and Highplex System. The analytical performance was evaluated to establish the limit of detection (LOD), precision and agreement.

There is an average difference in Ct of -0.883 and 0.078 between the nucleic extractors and MT-PCR systems, respectively. There was strong correlation (R-value ranging from 0.78 – 0.91 for all gene targets) between the AusDiagnostics Ultraplex-3 and Highplex. Bland Altman analysis demonstrated the overall differences between the assays are within the limits of agreement (LoA). The AusDiagnostics MT-Prep XL has a processing time of 1.04 minute/sample and AusDiagnostics Ultraplex 3, 1.93 minute/sample, demonstrating a higher efficiency rate compared to the reference standard.

RSV and SARS-CoV-2 detection in clinical samples were compared to the AusDiagnostics Highplex system as a reference standard. The assay demonstrated 99.10% overall agreement, with a PPA 100% and NPA of 99%. Kappa statistics of 0.95 ($p < 0.05$) suggests excellent agreement level between the two platforms. Moreover, precision study was conducted to observe the intra-run and inter-run assay variability. The calculated average Ct observed in all targets is 1.063% and 1.682%, respectively. The CV% is <2% suggesting high precision, low variability, and dispersion in data points.

The commercialized AusDiagnostics MT-Prep XL and Ultraplex 3 is a viable platform, fit for purpose in detecting the SARS-CoV-2, Influenza A, Influenza B and RSV respiratory viruses.

It's high efficiency rate and 96-sample capacity enhances laboratory throughput and enables faster time to result in comparison to the current reference standard.

Keywords: COVID-19; Influenza; Respiratory Syncytial Virus; Laboratory Diagnosis; MT-PCR

Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) which emerged from Wuhan, China, is a highly contagious virus that can cause fatal respiratory illness. Due to its highly transmissible nature, it has caused the current COVID-19 pandemic and posed a global public health concern [1,2]. It is important to

detect SARS-CoV-2 quickly to rapidly implement interventions to isolate the infectious patient, administer treatment and limit community transmission. Real-time reverse transcriptase PCR (rt-PCR) is a molecular diagnostic tool that is commonly used to accurately detect viral RNA. Therefore, emerging methods and technologies have been developed and exploited to reduce turnaround times, provide rapid diagnostic results, and increase laboratory throughput. This has been of high importance in times of surge testing, due to screening of symptomatic or asymptomatic patients, close or casual contact individuals, and routine surveillance of high-risk exposure individuals (e.g. healthcare and essential workers).

The AusDiagnostics MT-Prep XL Extractor and the AusDiagnostics Ultraplex-3 system have been recently developed with a capacity of 96 samples. The AusDiagnostics MT-Prep XL is an automated nucleic acid extractor integrated with Hamilton robotics, a 4-pipette channel liquid handling platform, and Thermofisher Kingfisher Presto technology, a sample purification system using the magnetic bead separation principle [3]. The AusDiagnostics Ultraplex 3 system is a 6-pipette channel processor and is based on multiplex tandem realtime PCR (MT-PCR) technology [4]. The commercialized AusDiagnostics MT-PCR assay detects the SARS-CoV-2 open reading frame *orf1a* and *orf8* gene targets and includes simultaneous detection of other common respiratory viral pathogens, Influenza A, Influenza B and Respiratory Syncytial Virus (RSV). These respiratory viruses are contagious and are commonly associated with seasonal outbreaks and hospitalizations of young children and elderly or comorbid patients [5-8]. Therefore, simultaneous detection assists with patient diagnosis and effective infection control [9,10].

Here, a validation study was performed to evaluate the analytical characteristics and performance of the AusDiagnostics MT-Prep XL viral nucleic extraction method and the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay (Ref. 80081) performed on the AusDiagnostics Ultraplex-3 system. This is aimed to determine whether the platforms and assay is fit for purpose in detecting SARS-CoV-2, Influenza A and B, and RSV in patient samples.

Methods

Study setting and study design

This study was conducted in the Department of Microbiology, Concord Repatriation and General Hospital, NSW Health Pathology, during March to July 2021. The laboratory serves a tertiary hospital, and during the study period, its two attached COVID-19 community testing centers. Samples from healthcare workers, hospital inpatients and outpatients were selected based on results of routine diagnostics testing with the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay, performed on the AusDiagnostics Highplex platform. These were re-tested on the AusDiagnostics Ultraplex-3 platform to compare analytical performance.

Specimen collection and storage

The samples for this study were collected from symptomatic/asymptomatic patients, SARSCoV-2 exposed patients, close or casual contacts and healthcare workers. Specimen types included a collection of nasal swabs, throat swabs, nasopharyngeal swabs (NP) and saliva swabs. Specimens were collected with flocked swabs transported in viral transport medium (VTM) and were store at 4°C prior for validation testing and processed within <7days of storage.

Nucleic acid extraction

Viral RNA was extracted on the AusDiagnostics MT-Prep XL (AusDiagnostics, Mascot, Australia) extractor, using the AusDiagnostics MT-Prep XL Viral Extraction Kit and Large 10 mL tube program. 300 µL of sample was extracted to an eluted volume of 50 µL, dispensed in an AusDiagnostics Ultraplex-3 96-deep well elution block. An AusDiagnostics extraction control was added in the lysis buffer (1 µL of extraction control per number of samples) prior to extraction. For validation of the AusDiagnostics MT-Prep XL, the performance is compared to the AusDiagnostics MT-Prep 24-extractor system (AusDiagnostics, Mascot, Australia), the reference laboratory standard extractor. Nucleic acid is extracted using the AusDiagnostics MT-Prep Viral Extraction Kit extracting 200 µL of sample to an eluted volume of 50 µL in 2 mL PCR flat bottom tubes.

Multiplex-tandem PCR and analysis

After RNA extraction, multiplex tandem polymerase chain reaction (MT-PCR) was performed using a commercial *in vitro* diagnostic (IVD) platform. 10 µL of sample elute extracted via the AusDiagnostics MT-Prep were run on the AusDiagnostics Highplex platform, whereas 20 µL of sample elute extracted via the AusDiagnostics MT-Prep XL were used on the AusDiagnostics Ultraplex-3 platform (as per manufacturer's instructions). See

Figure 1 for an overview of the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay workflow, performed on the AusDiagnostics Ultraplex 3. The extracted RNA sample, enzymes and master mixes are added to the AusDiagnostics Step 1 tubes containing lyophilised primers and probes for all gene targets (Figure 1A). This method involves a reverse transcription step and a pre-amplification step (15-18 cycles) to enrich all the gene targets. Subsequently, the amplified products are diluted into a 96-well dilution plate (Figure 1B). The diluted products are transferred into the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well Step 2 plates. Each well, contains nested primers specific for the gene target (Figure 1C). The Step 2 plate is then sealed, briefly spun, and immediately transferred to the AusDiagnostics MT-Analyser for the second PCR step, thermocycling at 95°C for 10minutes (1 cycle), 95°C for 10 seconds (30 cycles), 60°C for 20 seconds (30 cycles), 72°C for 10 seconds (30 cycles), 75°C for 5 seconds (50 cycles)) (Figure 1D).

The assay detects the gene targets for SARS-CoV-2a and b (*orf1a* and *orf8*), influenza A (H1, H3, H5 and H7), influenza A typing (pdH1N1, H3 and H3N2), influenza B (Yamagata and Victoria lineages) and RSV A and B. A human reference gene (NONO) detects human DNA and checks for sample adequacy. Moreover, an artificial sequence (Spike) is an internal control that checks for inhibition. In each PCR run, a positive control (AusDiagnostics Synthetic Respiratory Positive Control) and a negative control (Blank; DNase free water – No template control) is added to check for reliable detection of gene targets, sensitivity, and contamination.

The AusDiagnostics MT-Analyser Software provides automated curve interpretation.

Amplification of the target gene is reported as "Present", whereas a target gene is reported "Not detected" when there is no amplification that falls within predetermined parameters. In cases where cycling curve acceleration is slower than these parameters, it is reported as a "Check" result. This indicates that an operator's involvement is required to investigate and interpret the result. The software also provides calculated Ct values and target relative concentration expressed in arbitrary units, calculated relative to the internal control ("SPIKE") set at 10,000.

Evaluation and validation of the analytical performance of the AusDiagnostics MTPrep XL and the Ultraplex 3

22 patient saliva specimens and 21 patient NP swabs that have been previously tested negative for all the respiratory virus targets were selected for this validation study (n = 43). Samples were stored at 4°C prior to validation testing and processed within <7days of storage. 594 µL of patient sample were spiked with 6 µL of the AusDiagnostics Synthetic Respiratory positive control, totalling volume of 600 µL (1:100 dilution factor).

A 200 µL aliquot of the prepared spiked samples was extracted via the AusDiagnostics MT-Prep and run on the AusDiagnostics High-Plex. 300 µL of spiked samples underwent MTPrep XL extraction. Nucleic acid extracted via the AusDiagnostics MT-Prep XL were run both on the AusDiagnostics Highplex and AusDiagnostics Ultraplex-3 platforms. To compare the two extractors, the Ct values of samples extracted by the AusDiagnostics MT-Prep vs. AusDiagnostics MT-Prep XL and run on the AusDiagnostics Highplex were compared. Whereas to compare the two amplification platforms, the Ct values of samples extracted by the AusDiagnostics MT-Prep XL and run on the AusDiagnostics Ultraplex-3 were compared those from the AusDiagnostics Highplex platform. A correlation graph and Bland Altman graph was plotted to view concordance and agreement between the AusDiagnostics Ultraplex-3 and Highplex.

Time analysis study

The time efficiency (minutes/sample) was calculated for the AusDiagnostics MT-Prep XL and AusDiagnostics Ultraplex-3. The efficiency was compared to the reference method, AusDiagnostics MT-Prep 24 extractor and AusDiagnostics Highplex. This was calculated by dividing the total processing time by the sample capacity.

Positive (Sensitivity) and negative (specificity) agreement

A convenience sample of 334 patient specimens (nasopharyngeal swabs or saliva) were selected based on results of routine testing with the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay. The assay was run on the AusDiagnostics Ultraplex-3 system and was compared to the AusDiagnostics High plex platform as a reference. Percentage of positive and negative agreement analysis was used to describe sensitivity and specificity, and Cohen's kappa statistics was calculated to measure proportion of agreement.

Limit of detection

The AusDiagnostics Respiratory Synthetic positive control was diluted to 1, 1:10, 1:100, 1:1000, 1:2000, 1:4000 with DNase free water. 30 µL of this was added to the AusDiagnostics Ultraplex-3 and was tested in triplicate. The lowest relative concentration and highest Ct value for all gene targets was determined.

Precision

Intra-run variability evaluation

The intra-run assay precision was assessed by spiking 50 µL of a 1:10 dilution of the AusDiagnostics Respiratory Synthetic positive control in 400 µL of NP patient swabs (n = 9), that have been previously diagnosed as respiratory virus negative. Spiked samples were extracted via the MT-Prep XL Viral Extraction kit and run on the Ultraplex-3 in triplicate (N = 27). The average, standard deviation and %CV was calculated between the replicates for each sample. The average %CV for each sample was then calculated.

Inter-run variability evaluation

Inter-run assay variability was established by performing triplicates of a 1:3 dilution of the AusDiagnostics Respiratory Synthetic positive control on 22 runs on the Ultraplex-3, over the span of 11 days (n = 66), with varying technical operators. Average Ct for each positive control replicate in the run was calculated and the %CV was calculated between the runs.

Statistical analysis

All statistical analysis were calculated with Microsoft Excel software and Analyse-It (Microsoft, Redmond, USA). Overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) were calculated to describe sensitivity, specificity, and agreement. Cohen kappa statistics for the proportion agreement expected by chance (P_e) and proportion of agreement observed (P_o) was used to calculate K-value to determine agreement level between two platforms, with $p < 0.05$ showing significance. A correlation graph and a Bland Altman analysis using non-parametric measurements by calculating limits of agreement (LoA) as quantiles and establish median differences in Ct from clinical samples spiked with the AusDiagnostics Synthetic Respiratory positive control tested on the AusDiagnostics Ultraplex-3 and Highplex. The percentage of correlation of variation (CV%) was calculated to measure dispersion of data points between samples in a run (intra-run variability) and between each assay plate run (inter-run variability).

Figure 1: Schematic of the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay. A) A reverse transcription and a short PCR step occurs in the AusDiagnostics Step 1 tube, containing primers for all gene targets, SARS-CoV-2a (*orf1a*) and b (*orf8*), influenza A, influenza A typing, influenza B and RSV. B) Enriched amplified products are diluted, and C) aliquoted into each well of the AusDiagnostics Step 2 plate containing nested primers specific to the gene target. D) The Step 2 plate is transferred to AusDiagnostics MT-PCR Analyser for the second and final PCR step. The cycling data is captured, and an automatic qualitative interpretation and calculated Ct value is provided by the software.

Results

Evaluation and validation of the analytical performance of the AusDiagnostics MT214 Prep XL and Ultra-plex 3

Targets	Extractor comparison: AusDiagnostics MT-Prep XL vs. MT-Prep	Amplification comparison: AusDiagnostics Ultraplex-3 vs. Highplex
	Δ Ct (Av. Ct ± Std)	Δ Ct (Av. Ct ± Std)
Influenza A	-0.72 ± 1.28	0.04 ± 0.38
Influenza A Typing	-0.86 ± 1.16	0.14 ±0.39
Influenza B	-0.83 ± 1.02	-0.12 ± 0.33
RSV	-1.11 ± 1.04	0.09 ± 0.40
SARS-CoV-2 a	-0.79 ± 1.13	0.28 ± 0.42
SARS-CoV-2 b	-0.99 ± 1.11	0.04 ± 0.44

Table 1: The average Δ Ct value between spiked samples extracted on the MT-Prep XL vs. MT-Prep, and MT-Prep XL extracts run on the AusDiagnostics Ultraplex-3 vs. Highplex.

A comparative analysis between the AusDiagnostics MT-Prep XL and MT-Prep was conducted. Samples spiked with the Synthetic Respiratory positive control was extracted via the two methods and analysed on the AusDiagnostics High-plex system (control variable). The difference in Ct values shown an average change in Ct of -0.72 ± 1.28 for influenza A, 0.86 ± 1.16 for influenza A typing, -0.83 ± 1.02 for influenza B, -1.11 ± 1.04 for RSV, -0.79 ± 1.13 and -0.99 ± 1.11 for SARS-CoV-2-a and -b respectively (Table 2).

Targets	Extractor comparison: AusDiagnostics MT-Prep XL vs. MT-Prep	Amplification comparison AusDiagnostics Ultraplex-3 vs. Highplex
	Δ Ct (Av. Ct ±Std)	Δ Ct (Av. Ct ±Std)
Influenza A	-0.72 ± 1.28	0.04 ± 0.38
Influenza A Typing	-0.86 ± 1.16	0.14 ±0.39
Influenza B	-0.83 ± 1.02	-0.12 ± 0.33
RSV	-1.11 ± 1.04	0.09 ± 0.40
SARS-CoV-2 a	-0.79 ± 1.13	0.28 ± 0.42
SARS-CoV-2 b	-0.99 ± 1.11	0.04 ± 0.44

Table 2: The average Δ Ct value between spiked samples extracted on the MT-Prep XL vs. MT-Prep, and MT-Prep XL extracts run on the AusDiagnostics Ultraplex-3 vs. Highplex.

Validation of the AusDiagnostics Ultra-plex 3 was accomplished by comparing its Ct values (with samples spiked with the Synthetic Respiratory positive control) with those run on the Highplex. Nucleic acid was extracted via the AusDiagnostics MT-Prep XL. Differences in Ct values displayed an average change in Ct of 0.04, 0.14, -0.12, 0.09, 0.28 and 0.04 cycles respective to the gene targets (Table 1).

The concordance between the samples performed on the AusDiagnostics Ultraplex-3 compared to the Highplex was high for influenza A (r = 0.91), influenza A typing (r = 0.82), influenza B (r = 0.82), RSV (r = 0.76), SARS-CoV-2 a (*orf1a*) (r = 0.84) and b (*orf8*) (r = 0.78) (Figure 2). Although data demonstrates strong correlation, where 1 is equivalent to perfect correlation, it does not show whether the two data sets agree. Figure 3 shows a nonparametric

Bland Altman graph to compare the AusDiagnostics Ultraplex-3 a new method versus the current method AusDiagnostics Highplex. This was plotted by calculating the average Ct difference between the two platforms ($\frac{Ct_{Highplex}+Ct_{Ultraplex-3}}{2}$; x-axis) and 2 difference between measurements ($Ct_{Ultraplex-3} - Ct_{Highplex}$; y-axis). Majority of the data points for all targets are clustered near the median (red dashed line) and on average 41 out of 43 sample comparisons, the differences are smaller and within the LoA.

Figure 2. Correlation graph between Ct values of clinical samples spiked with the AusDiagnostics. Synthetic Respiratory positive control tested on the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay performed on the Ultraplex-3 and Highplex. The gene targets observed are (A) influenza A, (B) influenza A typing, (C) influenza B, (D) RSV, (E) SARS-CoV-2 a (*orf1a*) and (F) SARS-CoV-2 b (*orf8*).

Figure 3: Non-parametric Bland Altman diagram comparing the Ct values of clinical samples spiked with AusDiagnostics Synthetic Respiratory positive control tested on the AusDiagnostics SARS-CoV2, Influenza and RSV 8-well assay performed on the Ultraplex-3 compared to the Highplex. The gene targets observed are (A) influenza A, (B) influenza A typing, (C) influenza B, (D) RSV, (E) SARS-CoV2 a (*orf1a*) and (F) SARS-CoV-2 b (*orf8*). Lower and upper limits of agreement (LoA) calculated in quantiles (blue dashed lines) and median (red dashed lines).

Extraction Method	Sample capacity	Time (min)	Time (min)/ sample
AusDiagnostics MT-Prep XL	96	100	1.04
AusDiagnostics MT-Prep	24	58	2.41

Table 3: Comparison of the instrument time taken for different extraction methods, Aus Diagnostics MT-Prep XL vs. MT-Prep.

Processor	Sample capacity	Time (min)	Analyser Time (min)	Time (min)/ sample
AusDiagnostics Ultraplex-3	96	100	85	1.93
AusDiagnostics Highplex	24	98	72	7.08

Table 4: Comparison of the instrument time taken between eplex platforms, the 256 AusDiagnostics Ultraplex-3 vs. Highplex.

The run-time efficiency between extraction methods and eplex platforms was compared, to determine which system was more efficient and resulted in higher productivity. The AusDiagnostics MT-Prep XL has a sample capacity of 96 and takes 100 minutes to extract, or 1.04 min/sample (Table 3). The AusDiagnostics MT-Prep comparatively is 1.37 minutes slower per sample and has a capacity of 24 samples. The AusDiagnostics Ultraplex-3 has a processing time 1.93 min/sample, whereas, the AusDiagnostics Highplex has a significant difference of 5.15 min/samples, taking 7.08 min/sample (Table 4).

Method agreement

A convenience sample of 334 clinical patient samples were selected based on the results of the routine testing with the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay performed on the AusDiagnostics Highplex. These samples were then tested on the Ultraplex-3 for comparative evaluation. Positive and negative agreement was used to estimate sensitivity and specificity, respectively, as the true state of infection or disease in patients cannot be determined with confidence (Table 5). 331 out of 334 samples are concordant between the two platforms, with overall 99.10% agreement. 3 samples were falsely detected for RSV on the AusDiagnostics Ultraplex-3 platform, as this was determined by a negative result on the AusDiagnostics Highplex and high Ct values ranging from 32-33 cycles.

30 of the positive results were for the RSV target and 5 were for SARS-CoV-2 (both *orf1a* and *orf8*). The assay has a PPA of 100% and NPA 99% (Table 5). Cohen kappa (K) was 278 calculated to measure the degree of agreement between the AusDiagnostics platforms. The 279 P_e is 0.81, P_o is 0.99, with a calculated K-value of 0.95.

AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay	Agreement (n = 334) Number (%) [95% CI]	Positive agreement (sensitivity) (n = 35) Number (%) [95% CI]	Negative agreement (specificity) (n = 299) Number (%) [95% CI]
AusDiagnostics Ultraplex-3	331 (99.10%) [99.04-99.16%]	35 (100%) [NA]	296 (99.00%) [98.93-99.06%]

Table 5: Overall, positive, and negative agreement of the AusDiagnostics SARS CoV-2, 281 Influenza and RSV 8-well assay run on the AusDiagnostics Ultraplex-3 compared to the 282 AusDiagnostics High-plex (reference standard).

n = Total Sample Number; CI = Confidence Interval; NA = Not Available as confidence intervals cannot be calculated at 100%.

Limit of detection

The limit of detection (LOD) was determined by conducting serial dilutions of the AusDiagnostics Synthetic Respiratory positive control to find the lowest relative concentration of the target detected by the AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay performed on the AusDiagnostics Ultra-plex 3. Table 6 displays the lowest relative concentration and Ct values for the following targets.

AusDiagnostics SARS-CoV-2, Influenza, RSV 8-well assay Limit of Detection				
Gene Targets	Ultraplex-3		Highplex	
	Relative Concentration*	Ct	Relative Concentration	Ct
Influenza A	23	31.14	13	32.1
Influenza A Typing	7	33.1	8	32.3
Influenza B	6	33.21	6	33.35
RSV	7	32.99	10	33.4
SARS-CoV-2 a (*orf1a*)	7	32.87	13	33.12
SARS-CoV-2 b (*orf8)*	16	32	13	33.29

Table 6: Limit of detection (LOD) of the AusDiagnostics SARS-CoV-2, Influenza and RSV 8well assay by determining the lowest relative concentration of the SARS-CoV-2, influenza (A&B), and RSV targets tested on the AusDiagnostics Ultraplex-3 vs Highplex.

*relative concentration (in arbitrary units) is calculated relative to the internal control ("SPIKE") set at 10,000.

Precision

AusDiagnostics Ultra-plex 3 Precision		
AusDiagnostics SARS-CoV-2, Influenza and RSV 8well assay gene targets [Ref. 80081]	**Intra-run assay CV% (n = 66)**	**Inter-run assay CV% (n = 27)**
Influenza A	0.99	1.74
Influenza A Typing	1.18	1.53
Influenza B	1.00	1.65
RSV	1.24	1.64
SARS-CoV-2 a (*orf1a*)	0.96	1.77
SARS-CoV-2 b (*orf8*)	1.01	1.76

Table 7: AusDiagnostics Ultra-plex 3 precision analysis (coefficient of variation, CV%) of Ct values both Intra-run (n = 27) and Inter-run (n = 66) of AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay (Ref.: 80081).

Precision of the AusDiagnostics Ultraplex-3 was assessed by analysing intra-run and interrun variability. Summarised data are shown in table 7. Intra-run variability was tested on 9 NP patient samples spiked with a positive control (conducted in triplicate). The %CV for influenza A is 0.99%, influenza A typing 1.18%, influenza B 1.00%, RSV 1.24%, SARS-CoV2-a (*orf1a*) 0.96% and SARS-CoV-2-b (*orf8*) 1.01%. Inter-run variability was assessed by testing a positive control in triplicate in each Ultraplex-3 run over the span of 11 days, with a total 22 Ultraplex-33 runs (n = 66). The %CV calculated for the respective targets is 1.74%, 1.53%, 1.65%, 1.64%, 1.77% and 1.76%.

Discussion

The evaluation and validation of the AusDiagnostics MT-Prep XL (MT-Prep XL Viral Extraction kit) and Ultra-plex 3 (AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay [Ref 80081]). The extractor and extraction kit were compared to the AusDiagnostics MT-Prep 24-system Viral Extraction kit demonstrating an average difference in Ct of -0.883 across all gene targets, influenza (A&B), RSV and SARS-CoV-2 (*orf1a* and *orf8*). A negative value suggests a higher nucleic acid concentration was extracted on the MT-Prep XL. Whereas the assessment of the AusDiagnostics SARS CoV-2, Influenza and RSV 8-well assay was performed on the Ultraplex (Ref. 80081) compared to the High-plex (Ref. 20081). An average of 0.078 cycle difference was observed, while a strong correlation and agreement demonstrated that there are no significant differences between the analytical performances between the eplexes and platforms in detecting the gene targets.

The AusDiagnostics MT-Prep XL and the Ultraplex-3 system has a total processing time of 2.97 min/sample, and is more efficient than the reference laboratory standard, AusDiagnostics MT-Prep and Highplex system, of 9.49 min/sample. The reduced processing time of 6.52 min/sample in conjunction of increased sample capacity of 96, enables faster release of results, enhanced surveillance, and better clinical management.

The AusDiagnostics Ultraplex-3 demonstrated 99.10% OPA, 100% PPA and 99% NPA. The influenza gene targets were not able to be verified in the same manner, as insufficient patient samples were available due to low influenza activity in Australia in 2020 [11]. Using Cohen Kappa's statistical analysis, the K-value is 0.95. As the $0.81 < k < 1.00$, according to Cohen kappa's range, results show an excellent level of agreement between AusDiagnostics Ultraplex-3 and AusDiagnostics Highplex.

The LOD was assessed by conducting serial dilutions of the AusDiagnostics Synthetic Respiratory positive control. The AusDiagnostics SARS-CoV-2, Influenza and RSV 8-well assay (Ref. 80081) LOD was able to detect lower concentrations of the gene targets compared those specified in the IFU and overall lower when compared to the AusDiagnostics SARS CoV-2, Influenza and RSV 8-well assay (Ref: 20081) on the AusDiagnostics High-plex. However, the Highplex shows a slightly more sensitivity for influenza A and SARS CoV-2 b (*orf8*). However, a limitation of using this assay is that the concentration expressed outputted by the result software is relative to the internal control SPIKE and does not provide quantification of the viral load.

The reliability and precision of the output of results by the AusDiagnostics Ultra-plex 3 was assessed by analysing the intra-run and inter-run variability. The CV for all targets was below <2.5%, indicating that there is minimal variability and dispersion of data, between each sample in a run, and between each assay run, irrespective of plate batch number, reagents used, day, and technical operators. This demonstrates that the assay is reliable, and results are accurate, precise, and reproducible.

The limitations of this study include that there is limited availability of clinical respiratory positive specimens, and the number of samples were attributed to surveillance screening of health care workers. Therefore, a comparison can only be inferred using a spiking approach. Moreover, convenience samples were chosen for this validation study, hence, true sensitivity and specificity of the assay cannot be characterised. The applicability of the AusDiagnostics MT-Prep XL and Ultraplex 3, although more efficient than the reference standard, is only true depending on the laboratory and number of samples received at a time. As it would be inappropriate and contradict the purpose of the platforms, if in each MT-PCR run does not reach the 96-sample capacity and is used in laboratories where there is low number of testings. Time spent waiting to fill the 96-sample run or running few samples at a time would be costly and a wastage of resources and reagents. Therefore, this 96-sample nucleic acid extractor and MT-PCR platform is more suitable for laboratories processing large number of samples.

Conclusion

In conclusion, the analytical performance of the AusDiagnostics MT-Prep XL 96 extractor and AusDiagnostics Ultraplex-3 platform demonstrated excellent agreement compared to the reference laboratory standard, AusDiagnostics MT-Prep 24 extractor and AusDiagnostics High-plex platform. Moreover, it increases laboratory capacity and sample throughput, and is especially suitable for laboratories with high testing numbers. The evaluation of the performance parameters validated in this study shown that the AusDiagnostics SARS-CoV2, Influenza and RSV 8-well assay is fit for purpose and suitable in the detection of respiratory pathogenic viruses and screening of SARS-CoV-2.

Ngoc Yen Kim Tran: Methodology, Validation, Formal analysis, Investigation, Writing – Original Draft, Data Curation, Visualisation Thuy Phan: Methodology, Validation, Investigation, Supervision Genevieve Mckew: Writing – Review and Editing Steven Siarakas: Supervision, Project administration, Resources.

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Conflicts of Interest

The authors state that there are no conflicts of interest to disclose.

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