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SARS-CoV2 Antigen Assay Laboratory Evaluation Report:
Flowflex SARS-CoV-2 Antigen Rapid Test

Introduction

The Department of Molecular Medicine and Haematology is recognised by SAHPRA and the NHLS as an evaluation laboratory for SARS-CoV2 diagnostic assays and testing platforms.

Where possible relevant reference (standardised material) and residual clinical specimens will be utilized and obtained from patient specimens referred to the NHLS CMJAH PCR laboratories under University of the Witwatersrand HREC approval # M1911201.

To date, several laboratory validations have been performed on residual clinical specimens (PBS/VTM) collected during November 2020 to January 2021. The initial protocol (simulating swabs in residual clinical specimens) was created to resemble the manufacturer's instructions for use (IFU) under laboratory based conditions. However, an increase number of false negative results were observed when compared to the Ct values obtained from a standard of care RT-PCR assay. To eliminate false negative/positive results, the protocol was revised to adopt two strategies: (a) extend the evaluation panel (n=65 to n=110) to increase the data set (ensure robust accuracy testing) and (b) use residual clinical specimen with manufacturer supplied buffer in various dilutions (1:1 - 1:7) [(unless an alternative protocol for specimens stored in PBS/VTM was provided by the manufacturer)] and include storage media (PBS/VTM/UTM/saline) and nuclease-free water blanks to assess background detection. These strategies were based on extensive in-house troubleshooting/optimisation, literature review and direct communication with several manufacturers to establish an impartial protocol for evaluation under laboratory based conditions. Whereas, specimens stored in PBS/VTM deviates from the IFU, the collection of fresh specimens for each evaluation is not feasible for a laboratory based evaluation (patient enrolment, patient consent, seasonal variations in viral load etc.) and therefore, manufactures/suppliers may consider performing a clinical evaluation.

Antigen panel amendments

The Flowflex SARS-CoV-2 Antigen Rapid Test was initially evaluated using a total of 110 specimens [(SARS-CoV-2 high viral load (n=60), Ct <30, medium viral load (n=20), Ct 30-35 and SARS-CoV-2 negatives (n=30)]. However, a number of false positive and false negative results were observed in this assay. To further verify the integrity of our specimens, the same panel was tested on n=4 other SARS-CoV-2 rapid antigen test (including at least one rapid antigen test that has met the minimum acceptance criteria as a reference test). After reviewing the combined results, all consensus false positive and false negative specimens were removed from data analysis to omit potential errors attributable to specimen integrity. All the aforementioned modifications (panel extension, protocol amendments, data analysis) were made to ensure an unbiased fair evaluation under laboratory based conditions.

Disclaimer

This evaluation was conducted under the emergency use of diagnostics for COVID19, and the study was conducted with limited resources available at the time. Rapid assessment may not investigate all aspects of final diagnostic use. This document may be updated as and when additional information becomes available.

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Executive summary

Results

The Flowflex SARS-CoV-2 Antigen Rapid Test demonstrates a sensitivity of 95% and specificity of 100% on high viral load (Cycle threshold<30) specimens. The assay performance reduces to a sensitivity of 12% and specificity of 100% on medium viral load (Ct 30-35) specimens.

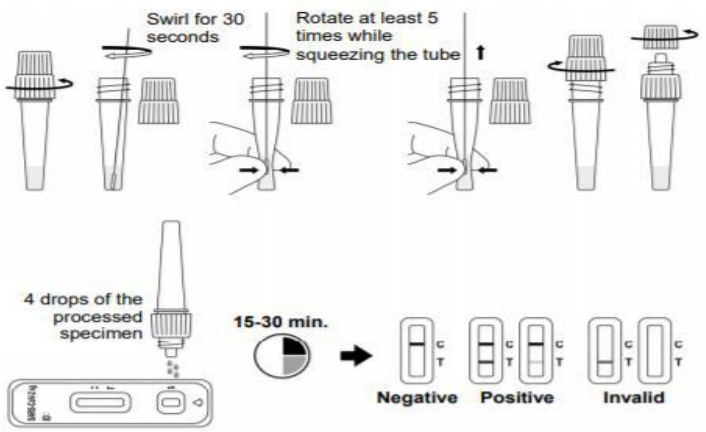
Challenges

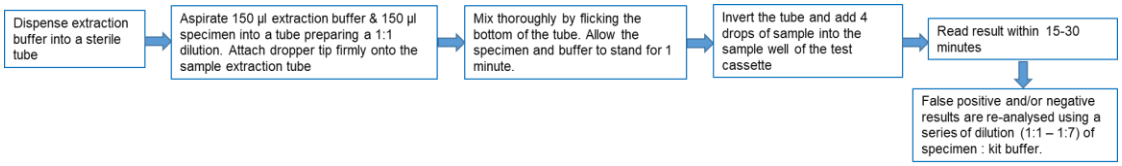
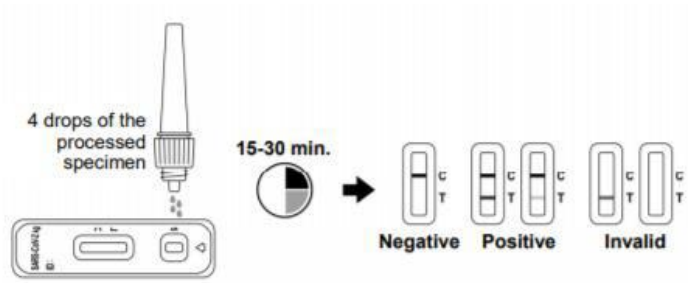
The test kit does not include certain consumables/materials (protective gloves, stop watch/timer, biohazard bin, spill kit) that are required for analysis.

Recommendations

The Flowflex SARS-CoV-2 Antigen Rapid Test may be recommended for use to diagnose infection with SARS-CoV-2 in patients with high viral load (Ct<30). Field performance and with end users was not evaluated.

Test Information

Type of Test	Antigen Lateral Flow Assay (LFA)
Assay name	Flowflex SARS-CoV-2 Antigen Rapid Test
Company name	ACON Biotech (Hangzhou) Co., Ltd.
International accreditation	CE-IVD
Brief test description	The Flowflex SARS-CoV-2 Antigen Rapid Test kit is an immunochromatographic assay for the qualitative detection of SARS-CoV-2 nucleocapsid protein antigen in human nasal and nasopharyngeal swab specimens. The test requires a visual read within 15-30 minutes of specimen incubation on the test device. The specimen testing process described by the manufacturer is as follows: **Method 1 (Manufacturers IFU):** Nasopharyngeal swab  Figure 1: Specimen testing flow (as per the kit Instructions for Use).

	Method 2 (in-house adapted protocol): Frozen VTM/PBS specimens (prepared using a 1:1 – 1:7 ratio of specimen with kit buffer)  Figure 2: Specimen testing flow for evaluation of Flowflex SARS-CoV-2 Antigen Rapid Test using specimen to kit buffer.
Result output and interpretation	 **INTERPRETATION OF RESULTS** (Please refer to the illustration above) **NEGATIVE:** Only one colored control line appears in the control region (C). No apparent colored line appears in the test line region (T). This means that no SARS-CoV-2 antigen was detected. **POSITIVE:*** Two distinct colored lines appear. One line in the control line region (C) and the other line-in the test line region (T). This means that the presence of SARS-CoV-2 antigen was detected. ***NOTE:** The intensity of the color in the test line (T) may vary depending on the level of the SARS-CoV-2 antigen present in the specimen. Therefore, any shade of color in the test line region (T) should be considered positive. **INVALID: Control line fails to appear.** Insufficient specimen volume or incorrect operation are the most likely reasons for control line failure. Review the procedure and repeat the test with a new test cassette. If the problem persists, discontinue using the test kit immediately and contact your local distributor. Figure 3: Interpretation of test results (as per the kit Instructions for Use).
Date of report	2nd July 2021
Report version	2.0 *(additional dilutions with supplier buffer [method 2] to address potential false positive results)*
Evaluation protocol	The **laboratory evaluation** measures analytical accuracy (sensitivity/specificity) on frozen residual clinical specimens. The specimens are selected based on the SARS-CoV-2 viral concentrations, which is extrapolated from the molecular PCR cycle threshold (Ct) value. Current literature and available guidelines[1] highlight Ag-RDTs most likely perform well in patients with Ct values<25. The Foundation for Innovative New Diagnostics include a further viral load range as Ct<33. The current protocol described here is based on the median Ct=30, which is derived from three months (November 2020 to January 2021) of laboratory test results reported within the National Health Laboratory Service corporate data warehouse. The challenge panel includes Ct<30 (considered high viral load) and Ct 30-35 (considered medium to low viral load). This Ct range is considered to correlate with known infectious virus[2] Assay precision (variability) is assessed by residual clinical specimens repeat testing. Standardized viral culture supernatant and purified recombinant proteins are used to semi-quantitatively measure limit of detection. Time-to-positivity (TTP) is recorded for all positive results.

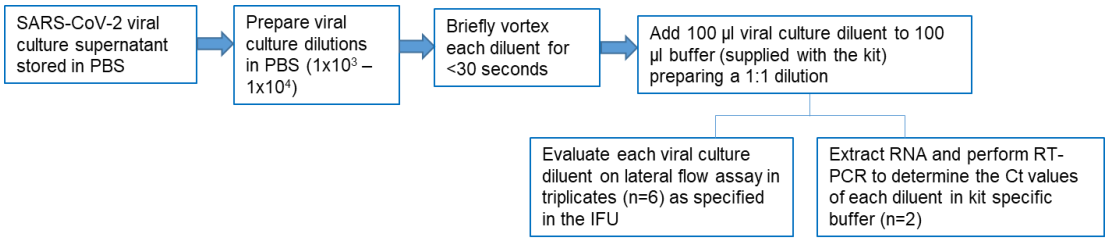
[1] WHO Antigen-detection in the diagnosis of SARS-CoV2 infection using rapid immunoassays. Interim guidance 11th September 2020

[2] La Scola, B et al (2020) European Journal of Clinical Microbiology & Infectious Diseases 39:1059-1061

	Laboratory Evaluation Panel **Residual clinical specimens** (n=110): SARS-CoV-2 detected, high viral load (n=60), Ct <30; SARS-CoV-2 detected, medium viral load (n=20), Ct 30-35; SARS-CoV-2 not detected (n=30); Blank control material/buffers: (PBS/VTM/UTM/Saline, n=4;Nuclease-free water, n=1) **Precision Data** (n=20): SARS-CoV-2 positive specimens (n=2), SARS-CoV-2 negative specimens (n=2) were tested five times on one day **Reference Material** Viral culture (n=8): Viral culture panel challenge (1:1000 & 1:10 000 diluents) in triplicate (n=6) compared to RT-PCR (n=2) **Reference Material** Recombinant proteins (n=16): Purified recombinant spike and nucleocapsid SARS-CoV-2 protein panels (10 nM – 2.4 pM) tested in duplicates N protein (n=12), S protein (n=4). **Qualitative assay performance** is assessed for the following criteria: (i) availability of all required materials in kit; (ii) ease of use; (iii) time to result (acceptable ≤40mins, desirable ≤20mins; (iv) invalid (error rate); (v) minimize the need for biosafety; (vi) minimal training required. Where applicable a score may be applied as such: Score: 1= very bad/difficult, 2= bad/difficult, 3= neutral, 4= good/easy, 5= very good/easy
Comparator technology	Comparator technology is based on standard of care (SOC) molecular SARS-CoV2 testing. The cobas® SARS-CoV-2 (Roche Molecular, Pleasanton, CA, USA) platform was used to determine the Ct values of clinical patient specimens. The qualification of viral culture supernatant was performed using the TaqMan® TaqPath™ COVID-19 CE-IVD RT-PCR Kit (multiplex) on QuantStudio (both ThermoFisher Scientific, Waltham, MA, USA).
Accuracy (sensitivity, specificity) and Agreement (Cohen Kappa coefficient)	Blank control material/buffers (PBS/VTM/UTM/Saline/Nuclease-free water) tested on the assay to determine any false positive results attributed to the formulation of storage media reported a false positive result on VTM and UTM (Appendix 1). Further dilution of the residual clinical specimens with supplier's buffer was therefore implemented prior to accuracy analysis Accuracy and agreement was measured using the following residual clinical specimens (n=100) with the following SOC characteristics: ➢ Cobas® SARS-CoV-2 positive specimens: n= positives (Ct range: 15,4-29,1 [n=57] and Ct 30,1-34,1 [n=17]) and n=26 negatives

VL concentration	n	Sensitivity/specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	Cohen Kappa (95% CI)	Agreement score
Method 2: Ct <30	83	95% (85-99)/ 100% (87-100)	100% (93-100)	90% (73-98)	0.92 (0.83-1.01)	Very good agreement
Method 2: Ct 30-35	43	12% (1.5-36)/ 100% (87-100)	100% (16-100)	63% (47-78)	0.14 (-0.04-0.32)	Poor agreement

Precision (reproducibility)	Precision data was determined in quadruplicates using the following panel: n=2 positive specimen and n=2 negative specimens. **All specimens were correctly identified** (refer to appendix 2).
Limit of detection	The manufacturer claims that the kit is able to detect up to $1.6x10^2$ $TCID_{50}$/mL (Median Tissue Culture Infectious Dose). The test LoD was investigated using viral culture supernatant. SARS-CoV-2 viral culture supernatants were diluted in 1 mL PBS to a final concentration of 1 in 1,000 and 1 in 10,000 (which approximates to log 5.9 and log 4.7 viral copies per milliliter). Equal volumes of viral culture diluent and kit specific buffer was prepared in quadruplicates for analysis on the lateral flow assay (n=3) and RNA extraction followed by RT-PCR (n=1).



2019-nCoV strain Tested	SARS-CoV-2 viral culture supernatant					
Dilution	Concentration in dilutions tested	RT-PCR			Flowflex SARS-CoV-2 Antigen Rapid Test	Call rate of replicates
		S	*N*	*ORF1ab*		
1/1000	Log 5.9	22,47	22,27	21,23	Positive	(3/3)
1/10000	Log 4.7	25,96	25,56	24,65	Positive	(3/3)
Limit of Detection (LOD)	The Flowflex SARS-CoV-2 Antigen Rapid Test could detect both viral culture diluents tested					

This assay could detect SARS-CoV-2 in both viral supernatant concentrations tested. To further establish the LoD; purified recombinant nucleocapsid (~60.68 kDa) and spike (~156.55 kDa) SARS-CoV-2 proteins that were expressed in Baculovirus expression system in insect cells[3] were tested. Although, the manufacturer states that the test line contains monoclonal antibodies against the N protein; the S protein was included to evaluate the specificity of the test. In order to determine the LoD a range of protein concentrations (10 nM – 2.4 pM) were prepared in a final volume of 150 µl using the Flowflex SARS-CoV-2 Antigen Rapid Test buffer supplied with the kit.

Material	Protein concentration	10 nM		2.5 nM		0.625 nM		0.039 nM		9.75 pM		2.4 pM	
Purified recombinant SARS-CoV-2 proteins	N Protein	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative
	S Protein	Positive	Positive	Positive	Positive	Positive	Positive	Negative	Negative	Negative	Negative	Negative	Negative
Limit of Detection (LoD): 0.039 nM N Protein													

Purified recombinant SARS-CoV-2 nucleocapsid protein was detected at ≥ 0.039 nM. Additionally, recombinant SARS-CoV-2 S protein was detected at ≥ 0.625 nM. However, since S protein was only detected at very high concentrations; this may have been due to weak binding affinity from protein-protein interaction at high protein concentrations. The LoD on the Flowflex SARS-CoV-2 Antigen Rapid Test was determined at 0.039 nM N protein.

Qualitative Performance

The qualitative performance is outlined as follows:

Characteristics	**Score (1-5)**	**Comments**
Availability of all required materials in kit	4	Does not include certain protective materials and consumables which will be required for field implementation.
Ease of use	5	IFU provides a detailed description for specimen collection, specimen preparation, testing process and result interpretation.
Time to result	5	A mean TTP was observed within 1 minutes range: (1-2 minutes).

[3] Purchased from Sengenics Corporation Pte Ltd, South Africa

	Invalid (error rate)	5	Control line was clearly visible for each test device.
	Minimize the need for biosafety	4*	The lateral flow test is relatively safe to use and can be executed without a biosafety laboratory, however biosafety disposal and spill kit will be required and ALL safety precautions during specimen collection and addition of clinical specimen to the test buffer/sample tube need to be adhered.
	Minimal training required	5	Basic standard procedure used for rapid antigen test. No instrument or specialised skills required.

**field evaluation not performed*

Recommendations

Acceptance criteria for laboratory performance of antigen RDT assays in identifying SARS-CoV-2 determined through this protocol design (under emergency use and in comparison to current in-country standard of care technology) is based on the following criteria: Note: In-country prevalence is not taken into account[4].

Acceptable criteria when the assays is performed on laboratory obtained specimens: Flowflex SARS-CoV-2 Antigen Rapid Test	***Level achieved by the assay evaluated***
≥97% specificity to minimize false positive results is acceptable	√
≥80% sensitivity to identify virus in high concentration is acceptable [5]	√
Identification of virus in low concentrations is desirable	*Some detection*
Assay generates a result within 40 minutes (acceptable)	√

Comments: The Flowflex SARS-CoV-2 Antigen Rapid Test assay demonstrated acceptable performance (95% sensitivity, 100% specificity) on specimens with high viral load. The Flowflex SARS-CoV-2 Antigen Rapid Test may be suitable for field evaluations.

[4] WHO TPP (31st July 2020): At low prevalence: PPV<<<50% would require 2nd test for confirmation, NPV is high. At 10-20% prevalence: PPV>75-98% and NPV still high (>95%)
[5] Zou.L *et al* (2020). SARS-CoV2 Viral Load in Upper Respiratory Specimens of infected Patients, NEJM, 382:12

Appendices: Additional information

Appendix 1: Statistical analysis.

Table 1: Raw data matrix for patient specimens –reference assay and target gene Ct values are shown.
Key: Green -SARS-CoV-2 negative, Red -SARS-COV-2 positive.

Specimen ID		Ct Values (Cobas)	Results	TTP
		E		
1	High Viral Load	15,4	Positive	1
2		15,9	Positive	1
3		16,2	Positive	1
4		16,7	Positive	1
5		16,8	Positive	1
6		17,1	Positive	1
7		17,2	Positive	1
8		17,3	Positive	1
9		17,3	Positive	1
10		17,4	Positive	1
11		17,5	Positive	1
12		17,5	Positive	1
13		17,5	Positive	1
14		17,6	Positive	1
15		17,8	Positive	2
16		17,9	Positive	1
17		17,9	Positive	1
18		18,1	Positive	1
19		18,2	Positive	1
20		18,3	Positive	1
21		18,3	Positive	2
22		19,2	Positive	2
23		19,2	Positive	1
24		19,3	Positive	1
25		19,3	Positive	1
26		19,4	Positive	2
27		19,4	Positive	1
28		19,5	Positive	1
29		19,5	Positive	1
30		19,6	Positive	2
31		19,6	Positive	1
32		20,1	Positive	2
33		20,1	Positive	1
34		20,2	Positive	1
35		20,2	Positive	1
36		20,6	Positive	1
37		20,6	Positive	2
38		20,6	Positive	1
39		20,7	Positive	2
40		21,2	Positive	2
41		21,3	Positive	1
42		23	Positive	2
43		23	Positive	2
44		23,4	Positive	1
45		23,4	Positive	1
46		23,5	Positive	1
47		24,1	Positive	2
48		24,1	Positive	1
49		24,1	Positive	2
50		24,3	Positive	2
51		24,3	Positive	1
52		25	Positive	2
53		26,7	Positive	2
54		26,8	Negative	
55		27,8	Negative	
56		29,1	Negative	
57		29,1	Positive	1

58	Medium Viral Load	30,1	Positive	1
59		31	Negative	
60		31	Negative	
61		32	Negative	
62		32,1	Positive	2
63		32,3	Negative	
64		32,3	Negative	
65		32,6	Negative	
66		32,6	Negative	
67		32,7	Negative	
68		32,7	Negative	
69		33	Negative	
70		33	Negative	
71		33,5	Negative	
72		34	Negative	
73		34	Negative	
74		34,1	Negative	
75	Negatives		Negative	
76			Negative	
77			Negative	
78			Negative	
79			Negative	
80			Negative	
81			Negative	
82			Negative	
83			Negative	
84			Negative	
85			Negative	
86			Negative	
87			Negative	
88			Negative	
89			Negative	
90			Negative	
91			Negative	
92			Negative	
93			Negative	
94			Negative	
95			Negative	
96			Negative	
97			Negative	
98			Negative	
99			Negative	
100			Negative	

Positive QC	Positive	
Negative QC	Negative	

Table 2: Precision data collected for 2 positive (Ct <30) and 2 negative specimens analysed in quadruplicates within the same day.
Key: Green -SARS-CoV-2 negative, Red -SARS-COV-2 positive

Specimen ID		Ct values (Cobas)	Results	TTP (min)
		E		
1a	Positive Specimen	24,1	Positive	2
1b			Positive	2
1c			Positive	2
1d			Positive	2
1e			Positive	2
2a	Positive Specimen	24,1	Positive	2
2b			Positive	2
2c			Positive	2
2d			Positive	2
2e			Positive	2
3a	Negative Specimen		Negative	
3b			Negative	
3c			Negative	
3d				
3e			Negative	
4a	Negative Specimen		Negative	
4b			Negative	
4c			Negative	
4d				
4e			Negative	

Table 3: Background detection from various storage media and nuclease-free water
Key: Green -SARS-CoV-2 negative, Red -SARS-COV-2 positive.

Storage media : kit buffer	Results
Viral Transport Media	Positive (faint band)
Phosphate Buffer Saline	Negative
Universal Transport Media	Positive (faint band)
Saline	Negative
Nucelase-free Water	Negative