

VALIDATION OF A MULTIPLEXED EXTERNAL CONTROL TO MONITOR SIMULTANEOUS DETECTION OF SARS-COV-2, FLU A/B AND RSV A/B IN VARIOUS NUCLEIC ACID TEST PANELS

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Introduction

Respiratory infections are among the most common viral illnesses, affecting millions of people annually. Influenza and RSV are leading global causes of hospitalization and death in older adults, those who are immunocompromised, and young children. In the SARS-CoV-2 post pandemic era, coinfection of SARS-CoV-2 with influenza or RSV has become a major public health concern.

Best practices and regulations require routine use of quality controls to monitor test systems for accuracy, shifts, trends, and random errors. Clinical labs require ready-to-use, non-infectious controls that provide a streamlined QC process to reliably confirm valid results and ensure that consumables and test systems perform as expected. A non-infectious synthetic control containing multiple gene sequences for SARS-CoV-2, Flu A/B, and RSV A/B has been developed and validated for use across multiple platforms to enable confidence and accuracy of patient results.

Materials and Methods

The Birlinn CoV2-Flu-RSV Control Panel contains multiple conserved gene sequences for SARS-CoV-2, Flu A/B, and RSV A/B detectable by nucleic acid tests targeting these gene segments. Sequences were designed *in silico*, synthesized, and cloned to create stable frozen clone stocks. In vitro RNA transcripts were generated, quantified, and formulated in a proprietary stabilizing, protective matrix. Validation studies demonstrated run-to-run and within-run precision across multiple platforms. Performance studies, including failure mode, shipping, and stability, were conducted to show robustness and reliability.

Figure 1. Design Strategy to create the Birlinn Control

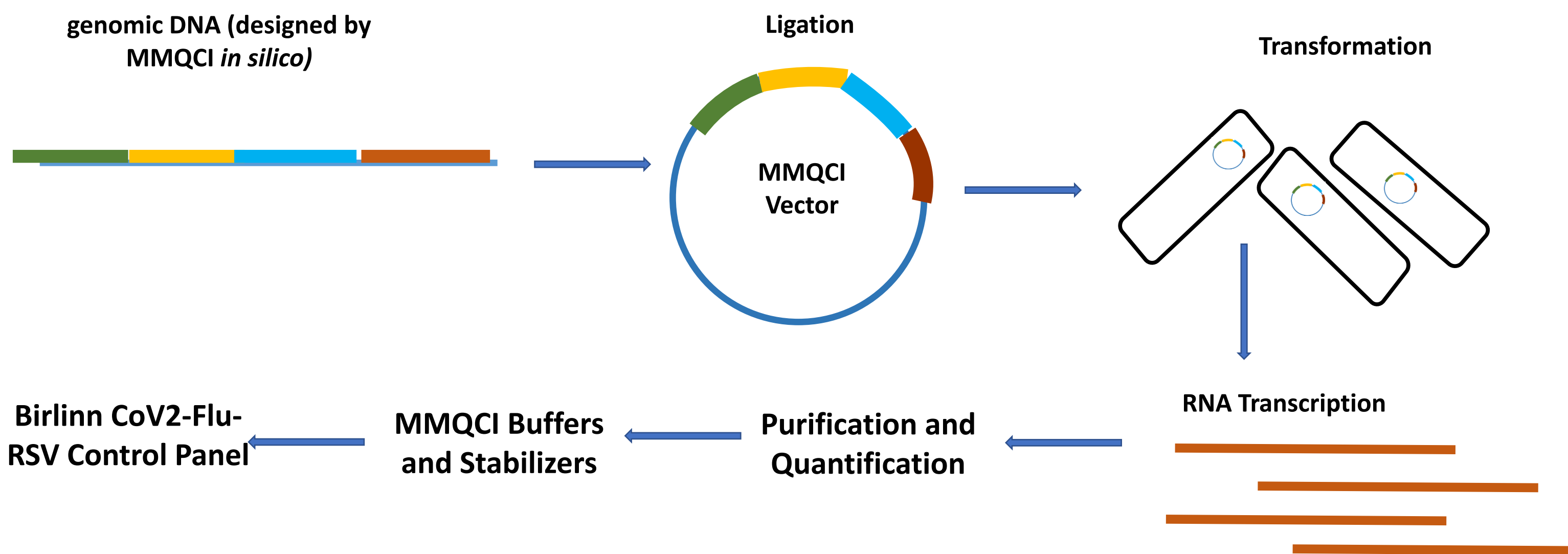


Table 1. Gene Sequences included in Birlinn CoV2-Flu-RSV Positive

Table 1: Birlinn CoV2-Flu-RSV Positive Analytes	
Analyte	Target Gene Sequences
SARS-CoV-2	S gene (Spike Surface Glycoprotein)
	E gene (Small Envelope Protein)
	M gene (Matrix Protein)
	ORF7ab
	ORF8
	N gene (Nucleocapsid)
	ORF 10
	ORF 3a
	RdRP (RNA-dependent RNA Polymerase)
Influenza A	PA gene (Polymerase Acidic Protein)
	M gene (Matrix Protein)
	PB2 gene (Polymerase Protein)
Influenza B	M gene (Matrix Protein)
	NS1 & NS2 gene (Non-structural protein)
Respiratory Syncytial Virus A	N gene (Nucleocapsid)
	M gene (Matrix Protein)
	M2 gene (Matrix 2 Protein)
Respiratory Syncytial Virus B	N gene (Nucleocapsid)
	M gene (Matrix Protein)
	M2 gene (Matrix 2 Protein)
Human RNase P	RNase P gene

Table 2. Gene Sequences included in Birlinn CoV2-Flu-RSV Negative

Table 2: Birlinn CoV2-Flu-RSV Negative Analyte	
Analyte	Target Gene Sequences
Human RNase P	RNase P gene

Results

Internal and External validation testing of Birlinn CoV2-Flu-RSV Control Panel

A total of 312 samples were tested. Of the 312 samples, 144 were tested for SARS-CoV-2 using RT-qPCR (Roche LC480 II, Table 3), and 56 samples were tested using Xpert Xpress CoV-2/Flu/RSV *plus* on the GeneXpert System (Table 4). 112 samples were tested externally across 5 different sites, incorporating seven different diagnostics platforms and four separate laboratory developed Real-Time qPCR assays (Table 7).

Table 3. Summary of Internal Testing on SARS-CoV-2 qPCR Assay

144 samples were extracted with QIAamp Viral RNA Extraction kit and run on the LC480 II system using a validated RT-qPCR assay for detection of SARS-CoV-2 N1 gene. Of the 144 controls tested, all had correct calls with valid results, and a %CV of less than 5% for all reported Ct values across all positive lots.

Internal Testing summary data with SARS CoV-2 qPCR assay							
P/N	Control	Lot #	No of Tests	Result	Ave Ct	SD	%CV
M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	24	Positive	31.0	0.3	1%
M51046	Birlinn CoV2-Flu-RSV Positive	F13MAR25A	24	Positive	30.9	0.3	1%
M51046	Birlinn CoV2-Flu-RSV Positive	F14APR25A	24	Positive	30.7	0.5	2%
M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	24	Negative	0.0	0.0	0.0
M51146	Birlinn CoV2-Flu-RSV Negative	H12MAR25A	24	Negative	0.0	0.0	0.0
M51146	Birlinn CoV2-Flu-RSV Negative	E11APR25A	24	Negative	0.0	0.0	0.0
Total tests			144				

Table 4. Summary Table of Internal Testing on Xpert Xpress CoV-2/Flu/RSV *plus*

56 total samples were tested on three different reagent lots of Xpert Xpress CoV-2/Flu/RSV *plus* on the GeneXpert System. Of the 56 controls tested, all 56 results were valid with 100% correct calls, and a %CV of less than 5% for all reported Ct values across all positive lots.

Internal Testing summary with Xpert Xpress CoV-2/Flu/RSV plus										
P/N	Control	Lot #	No of Tests	Expected Results	CoV-2 Ave Ct	Flu A1 Ave Ct	Flu A2 Ave Ct	Flu B Ave Ct	RSV Ave Ct	Percent Correct
M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	16	Positive	27.4	22.9	24.8	22.5	23.7	100%
M51046	Birlinn CoV2-Flu-RSV Positive	F13MAR25A	6	Positive	26.9	22.2	24.4	22.2	23.5	100%
M51046	Birlinn CoV2-Flu-RSV Positive	F14APR25A	6	Positive	26.6	21.7	24.0	21.8	22.8	100%
M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	16	Negative	0.0	0.0	0.0	0.0	0.0	100%
M51146	Birlinn CoV2-Flu-RSV Negative	H12MAR25A	6	Negative	0.0	0.0	0.0	0.0	0.0	100%
M51146	Birlinn CoV2-Flu-RSV Negative	E11APR25A	6	Negative	0.0	0.0	0.0	0.0	0.0	100%
Total tests			56	Avg of Pos	27.0	22.3	24.4	22.2	23.3	
				SD	0.4	0.6	0.4	0.3	0.5	
				% CV	1%	3%	2%	2%	2%	

Table 5. Evaluation of Failure Modes using Birlinn CoV2-Flu-RSV Positive control

Failure modes studies were completed to confirm the Birlinn CoV2- Flu-RSV control samples would alert an end-user to errors in sample handling, or system errors, by mimicking operator error. An incorrect sample volume of the Birlinn CoV2-Flu-RSV Positive control and a simulated clinical sample were added to the Xpert Xpress CoV2/Flu/RSV *plus* cartridges. All runs performed under failure mode analysis were reported as invalid runs for both Birlinn CoV2-Flu-RSV Positive control and the simulated clinical samples.

Tested Correctly		Addition of Incorrect Sample Volume into Cartridges	
Birlinn CoV2-Flu-RSV Positive	Simulated clinical Sample	Birlinn CoV2-Flu-RSV Positive (n=3)	Simulated clinical Sample (n=3)
Detected	Detected	Invalid	Invalid

Table 6. Shipping Study Testing Results

Birlinn CoV2-Flu-RSV Control Panel kits were placed on dry ice and gel packs following standard shipping configurations and maintained for 12 days at ambient temperature, to ensure dry ice had been depleted and gel packs no longer maintained refrigerated temperatures. To simulate a shipping delay, samples were exposed to three temperature fluctuation events and tested after 2 days at ambient temperature with 1 freeze/thaw or 1 refrigerate/warming event, and again after 12 days, incorporating a total of three freeze/thaw or refrigerated/warming events. All samples were tested with Xpert Xpress CoV-2/Flu/RSV *plus* assay with no significant difference from pre-shipping conditions.

Analytes	Standard Shipping Study			Shipping Delay Study			
	Pre-shipping (n=3)	Dry Ice (n=3)	Gel packs (n=3)	After exposure to shipping on dry ice		After exposure to shipping on gel packs	
				2-day delay (n=3)	12-day delay (n=3)	2-day delay (n=3)	12-day delay (n=3)
	Avg Ct	Avg Ct	Avg Ct	Avg Ct	Avg Ct	Avg Ct	Avg Ct
SARS-CoV-2	27.1	26.8	27.0	27.1	27.2	26.9	27.5
Flu A 1	22.3	22.1	22.2	22.4	22.8	22.3	22.6
Flu A 2	24.5	24.3	24.5	24.7	25.2	24.5	24.9
Flu B	22.3	22.1	22.1	22.3	22.6	22.3	22.4
RSV	23.5	23.4	23.5	23.6	24.0	23.5	23.7

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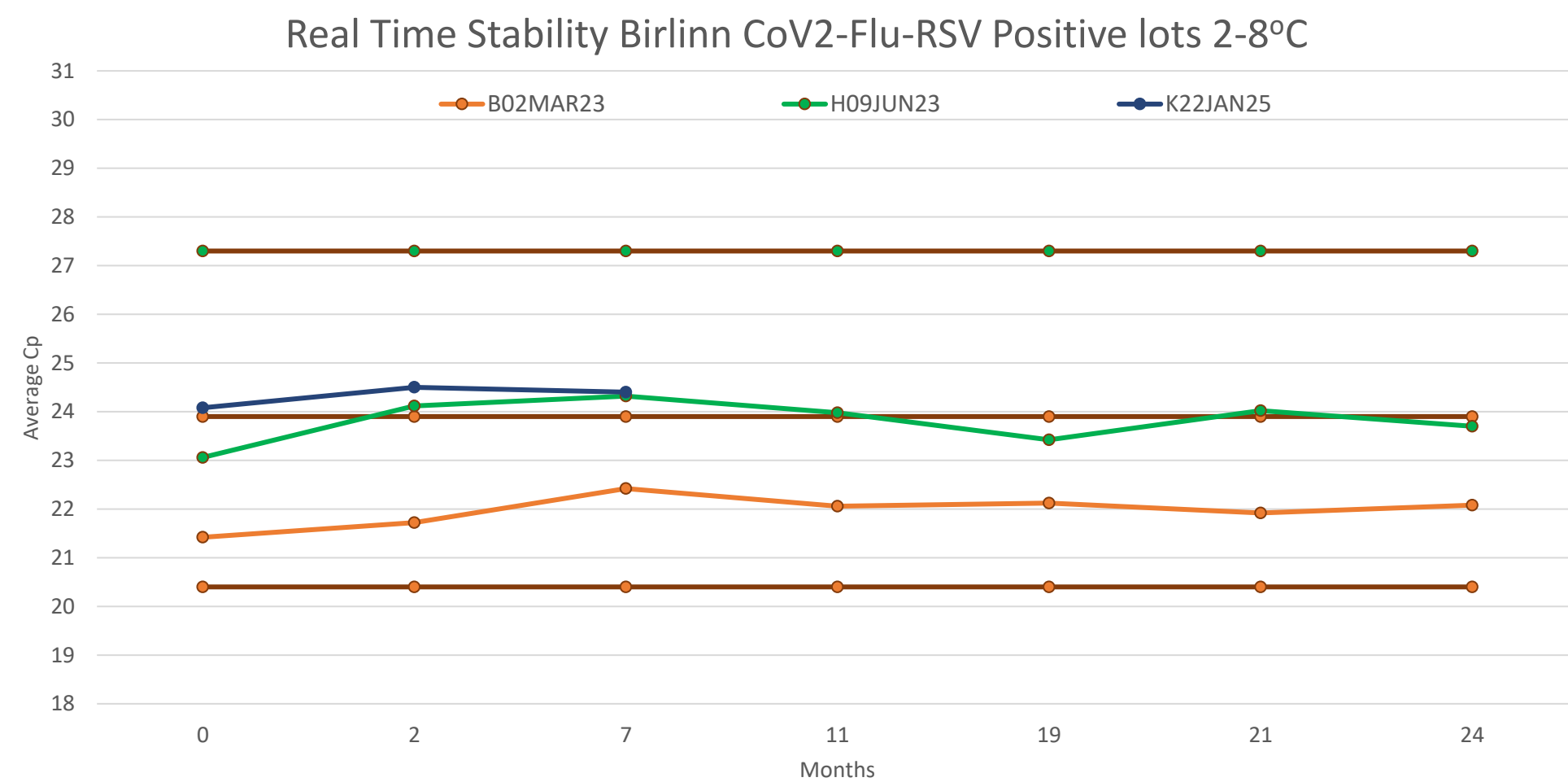
Table 7. Summary of External Testing

Birlinn CoV2-Flu-RSV Control Panel was tested externally on seven different diagnostics platforms including, Alinity m SARS-CoV-2 assay, cobas® Influenza A/B & RSV assay, cobas® SARS-CoV-2 & Influenza A/B assay, BD Respiratory Viral Panel, BD SARS-CoV-2 reagent, Panther Fusion® SARS CoV-2 assay and Panther Fusion® SARS CoV-2/Flu A/B/RSV assay, and four separate laboratory developed Real-Time qPCR assays, extracted with Thermo Scientific KingFisher Flex. Of the 112 samples tested, all had 100% correct calls with valid results.

External Validation Testing of Birlinn CoV2-Flu-RSV Control Panel											
Assay Name	P/N	Control	Lot #	No of Tests	Expected Results	CoV-2 Ave Ct	Flu A Ave Ct	Flu B Ave Ct	RSV Ave Ct	RNase P Ave Ct	Percent Correct
Alinity SARS CoV-2 Assay	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	2	Positive	24.5 (Positive)	N/A	N/A	N/A	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	2	Negative	0.0 (Negative)	N/A	N/A	N/A	N/A	100%
cobas Liat Influenza A/B & RSV assay	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	N/A	Detected	Detected	Detected	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	N/A	Not Detected	Not Detected	Not Detected	N/A	100%
cobas Liat SARS-CoV-2 & Influenza A/B assay	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	Detected	Detected	Detected	N/A	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	Not Detected	Not Detected	Not Detected	N/A	N/A	100%
BD Respiratory Viral Panel	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	Pos	Pos	Pos	Pos	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	Neg	Neg	Neg	Neg	N/A	100%
BD SARS-CoV-2 Reagent	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	Pos	N/A	N/A	N/A	Pos	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	Neg	N/A	N/A	N/A	Pos	100%
Panther Fusion® SARS-CoV-2 assay	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	3	Positive	POS/27.0	N/A	N/A	N/A	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	3	Negative	Neg	N/A	N/A	N/A	N/A	100%
Panther Fusion® SARS-CoV-2/Flu A/B/RSV assay	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	3	Positive	POS/27.0	POS/ 26.6	POS/27.3	POS/25.5	N/A	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	3	Negative	Neg	Neg	Neg	Neg	N/A	100%
SARS-CoV-2 Real-Time PCR	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	26.5	N/A	N/A	N/A	28.8	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	0.0	N/A	N/A	N/A	21.5	100%
Influenza A Real-Time PCR	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	N/A	23.4	N/A	N/A	27.9	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	N/A	0.0	N/A	N/A	20.7	100%
Influenza B Real-Time PCR	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	N/A	N/A	23.3	N/A	27.9	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	N/A	N/A	0.0	N/A	20.7	100%
RSV Real-Time PCR	M51046	Birlinn CoV2-Flu-RSV Positive	K22JAN25A	6	Positive	N/A	N/A	N/A	26.6	33.5	100%
	M51146	Birlinn CoV2-Flu-RSV Negative	E30OCT24A	6	Negative	N/A	N/A	N/A	0.0	26.2	100%
Total tests				112							

Figure 2: Three lots of Birlinn CoV2-Flu-RSV Control Panel were stored at 2-8°C and tested over 24 months using Xpert Xpress CoV-2/Flu/RSV *plus*. All lots across all time points resulted in 100% correct calls.

Linear regression was performed for all three positive lots, and no significant trend or shift was observed. Testing is ongoing.



Conclusion

Testing across multiple extraction technologies, various diagnostic assays and common clinical instruments demonstrated the ability of Birlinn CoV2-Flu-RSV Control Panel to support accurate testing and the utility as a ready-to-use, non-infectious control that could provide a streamlined QC process to reliably confirm valid results and ensure that consumables and test systems perform as expected.

The multiplex external control provides a stable, non-infectious, easy solution for training, verification, and routine monitoring of the performance of various comprehensive, integrated molecular assays.