Update of Influenza RT-qPCR Assays

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Introduction

The global SARS n-CoV-2 pandemic has demonstrated the need for pandemic preparedness and continuous infectious disease surveillance. MRIGlobal has had a quarterly influenza readiness program in place for many years, in which a RNA extraction and RT-qPCR assay is performed quarterly. The program currently uses the Centers for Disease Control and Prevention (CDC) protocols to detect circulating influenza strains of interest. The primers and probes needed to be updated to reflect more current strains, especially due to the high rate of re-assortment in influenza's viral genome. The World Health Organization (WHO) maintains a global surveillance system that tracks the circulating strains and detects mutations from season to season. Following WHO updates, MRIGlobal has been working to update our current RT-qPCR assays for influenza. To do this, the primers and probes used to detect our current agents of interest; Strain A, Strain B1 and Strain B2 were all updated, and a new strain of interest was also tested and added to the panel, Strain C. This study follows the JPEO-QA Program Standard Procedure, *New Methods Acceptance Requirements*, by completing limit of detection (LOD), near neighbor, acceptance, and demonstration of capability studies. The final side-by-side testing will be completed to demonstrate at least a same level of effectiveness of the updated assay with the current. The updated assay will be used to maintain technical readiness with MRIGlobal staff, and will serve as an at-the-ready capability, that could be called upon for expanded client needs.

Initial Testing

Before the start of the Limit of Detection study, an initial range finding test needed to be performed. Dilutions of the synthetic RNA for each target were made at the following concentrations and run in duplicate: 10ng/ul, 1ng/ul, 0.1ng/ul, 10pg/ul, 1pg/ul, and 10fg/ul. The cycle thresholds (Ct) for each assay at the lowest concentration (10fg/ul) were in the 24 – 26 range, demonstrating high sensitivity.

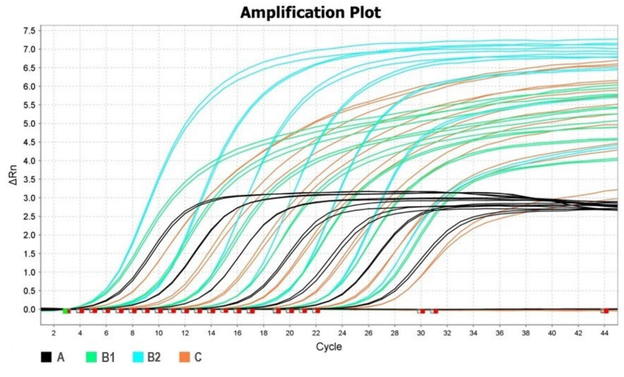

Image 1 – Initial Testing of each assay with concentrations ranging from 10ng/ul to 10fg/ul tested in duplicate.

With strong initial data, the cycling conditions were revisited to potentially shorten the run time. The total run time for the current CDC assays are about an hour while the WHO suggested cycling conditions left a total run time of around 90 minutes. Comparing the length of time for the reverse transcription steps between the 2 cycling conditions, the time was decreased from 20 minutes to 5 minutes and 30 seconds. There was no change in the Ct values with this decrease in time. The denaturation and extension times were also compared between the 2 protocols and the time was shortened from 15 seconds to 3. This change was also tested with the new primers and probes, and there was a slight increase of about 0.3 Ct across the 4 singleplex assays. The benefit of the shortened run time without a large shift in sensitivity led to the decision to keep the shortened cycling conditions.

Limit of Detection Determination

To determine the limit of detection (LOD) for each of the assays, a broad range of concentrations were tested in replicates of 10 and over a 4-log range. The lowest concentration to have 10 out of 10 positives (amplification with a Ct less than 40) was designated as the Broad Range LOD.

Broad Range LOD Testing				
Assay	**1 fg/ul**	**0.1 fg/ul**	**0.01 fg/ul**	**0.001 fg/ul**
A	10/10	10/10	**10/10**	7/10
B1	10/10	**10/10**	0/10	0/10
B2	10/10	**10/10**	9/10	4/10
C	10/10	10/10	**10/10**	8/10

Table 1 – Broad Range LOD testing for A, B1, B2, and C assays. The table shows how many of the 10 replicates resulted in positive amplification for each concentration tested.

After the Broad Range LOD was determined, four new concentrations within a 1-log range were tested in replicates of 10, with the highest concentration being the Broad Range LOD. The lowest concentration to have 10 out of 10 positives in this Narrow Range test is the established limit of detection for that assay.

Narrow Range LOD Testing				
Assay	**Concentration Tested**			
A	0.1fg/ul	0.06fg/ul	**0.03fg/ul**	0.01fg/ul
	10/10	10/10	10/10	8/10
B1	0.02 fg/ul	0.01 fg/ul	**0.005 fg/ul**	0.001 fg/ul
	10/10	10/10	10/10	5/10
B2	0.1fg/ul	**0.06fg/ul**	0.03fg/ul	0.01fg/ul
	10/10	10/10	5/10	0/10
C	0.1fg/ul	**0.06fg/ul**	0.03fg/ul	0.01fg/ul
	10/10	10/10	8/10	2/10

Table 2 – Narrow Range LOD testing for A, B1, B2, and C assays. The table shows how many replicates resulted in positive amplification for each concentration tested.

Assay	Final Limit of Detection
A	0.03 fg/ul
B1	0.02 fg/ul
B2	0.06 fg/ul
C	0.06 fg/ul

Table 3 – Final Limit of Detection concentrations for each assay.

For the B1 assay, the LOD was determined to be 0.005fg/ul, however during early validation testing there were a few false positives. The Narrow Range LOD was repeated for this assay and the new LOD was determined as 0.02fg/ul.

Near Neighbor Testing

A near neighbor study was conducted to screen for any cross-reactivity between the targets and assays. A single spike of each target of interest was tested for all 4 assays at double the established LOD. The targets were tested in replicates of 4 for assays not specific to their sequences, and in replicates of 2 for their specific assays. Neither assay showed amplification for the non-specific RNA.

RNA Type	A	B1	B2	C
Assay "A"	2/2	0/4	0/4	0/4
Assay "B1"	0/4	2/2	-	0/4
Assay "B2"	0/4	-	2/2	0/4
Assay "C"	0/4	0/4	0/4	2/2

Table 4 – The table shows the number of positive replicates out of the number of replicates tested. The B1 and B2 assays were not tested against each other due to the high similarity in sequences. Some cross-reactivity is expected between the 2 clades.

Assay Validation and DOC

Each assay was validated with 15 known samples of synthetic RNA spiked extraction buffer and 15 blank samples of extraction buffer. The study required 2 operators to run the same set of samples, for a total of 60 samples tested for each assay. The average Ct values of the positive samples at a threshold of 0.1 ΔR across the two operators, as well as the standard deviation across all the positive samples were determined. No false negatives nor false positives were observed.

Validation Testing		
Assay	**Ct Values**	
A at 0.03 fg/ul	Average	35.516
	St Dev.	0.541
B1 at 0.02 fg/ul	Average	35.189
	St Dev.	0.655
B2 at 0.06 fg/ul	Average	33.599
	St Dev.	0.480
C at 0.06 fg/ul	Average	34.751
	St Dev.	0.978

Table 5 – Validation testing for A, B1, B2, and C assays. The table shows the average Ct values of each assay across both operators, as well as the standard deviation for each assay.

A demonstration of capability (DOC) test was conducted for each assay. There were 30 blinded samples of extraction buffer, 15 of which were spiked with the LOD concentration of synthetic RNA and 15 left un-spiked. The samples were run by 2 operators, leaving a total of 60 samples tested for each assay. The average Ct values of the positive samples at a threshold of 0.1 ΔR across the two operators, as well as the standard deviation across all the positive samples were determined.

DOC Testing			
Assay	**Positive**	**Ct Values**	
A at 0.03 fg/ul	15/15	Average	35.181
		St Dev.	0.410
B1 at 0.02 fg/ul	15/15	Average	35.624
		St Dev.	0.720
B2 at 0.06 fg/ul	15/15	Average	31.902
		St Dev.	0.523
C at 0.06 fg/ul	15/15	Average	33.465
		St Dev.	0.787

Table 6 – DOC testing for A, B1, B2, and C assays. The table shows the average Ct values of each assay across the operators, as well as the standard deviation for each assay and the positivity rate of the unblinded positive results.

Future Testing

Due to the nature of handling influenza virus, a modified side-by-side comparison of the updated and current assays will be performed. To demonstrate the effectiveness of the new primers and probes in the readiness testing structure, an RNA extraction will be performed by operators and these extracts will be run across all assays in triplicate. All samples should be negative when run with both the current and updated assays, and the extraction control should be positive. These results, along with the completed development testing, will demonstrate the effectiveness of the new assays, and they will be integrated into operational use by MRIGlobal staff.

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