

**WHO External Quality Assessment Programme for the  
Detection of Influenza Virus Type A by PCR**

**Summary Report**

**Panel 6 (June-August 2009)**

**Virology Division  
Public Health Laboratory Services Branch  
Centre for Health Protection  
Department of Health  
Hong Kong**

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## 1. Introduction

The current outbreak of influenza A(H1N1)v virus [henceforth: 2009 pandemic influenza A (H1N1) virus is referred to as influenza A(H1N1)v virus, where v stands for variant] was first detected in Mexico in late March 2009. To assess the proficiency of RT-PCR testing and monitor the performance of laboratories in detecting influenza A viruses including the novel influenza A(H1N1)v virus, Panel 6 of the External Quality Assessment Programme (EQAP) consisting of 10 EQAP samples was dispatched between June and August 2009. For laboratories that requested positive controls, four samples of vacuum-dried influenza A virus RNA, A/Hong Kong/845/08 (H1N1), A/Hong Kong/918/08 (H3N2), A/Hong Kong/2090/2009 (H1N1v) and A/Duck/Singapore/F119-3/97 (H5N3) were included in the shipment together with instruction on how to prepare “in-house positive control” for nucleic acid amplification using the provided dried RNA. This report summarizes results reported by participating laboratories and analyzed them in light of various methodologies used in participating laboratories.

## 2. Panel description

In this dispatch, each laboratory received a panel of 10 coded samples and a vial of reconstitution buffer, all the 10 samples contained virus RNA (Table 1). Six samples contained vacuum-dried H5N1 virus RNA from three strains representing viruses from genetic cluster of clade 2.1, clade 2.2 and clade 2.3.4. While the concentration of clade 2.2 strain in the two samples had 1 log<sub>10</sub> difference, the concentration of clade 2.1 and clade 2.3.4 strains in the two samples were similar. The concentrations of clade 2.1 strain in the samples were 1 log<sub>10</sub> lower than the concentration of clade 2.3.4 strain. For the remaining four samples, one sample contained A/Brisbane/59/2007-like H1N1 virus RNA, one sample contained A/Brisbane/10/2007-like H3N2 virus RNA and two samples contained the influenza A (H1N1)v virus with 1 log<sub>10</sub> difference in concentrations between the two samples.

Table 1. The characteristics of the 10 samples

Sample No.	Strain	Clade <sup>a</sup>	Copies/ $\mu$ L <sup>b</sup>
2009-11	H5N1	2.3.4	1.176 x 10 <sup>3</sup>
2009-12	H1N1v	A/California/4/2009-like	2.037 x 10 <sup>2</sup>
2009-13	H1N1 (isolated in 2009)	A/Brisbane/59/2007-like	7.933 x 10 <sup>3</sup>
2009-14	H5N1	2.2	1.690 x 10 <sup>3</sup>
2009-15	H5N1	2.3.4	1.970 x 10 <sup>3</sup>
2009-16	H1N1v	A/California/4/2009-like	4.560 x 10 <sup>1</sup>
2009-17	H5N1	2.1	3.380 x 10 <sup>2</sup>
2009-18	H3N2 (isolated in 2009)	A/Brisbane/10/2007-like	2.440 x 10 <sup>3</sup>
2009-19	H5N1	2.1	4.727 x 10 <sup>2</sup>
2009-20	H5N1	2.2	1.876 x 10 <sup>4</sup>

<sup>a</sup> The nomenclature of H5N1 strains was based on the HA genes of H5N1 viruses (30 September 2009, date last accessed):

[http://www.who.int/csr/disease/avian\\_influenza/guidelines/nomenclature/en/index.html](http://www.who.int/csr/disease/avian_influenza/guidelines/nomenclature/en/index.html)

<sup>b</sup> Measured by real-time PCR after 5 days of storage of viral RNA at 25°C.

### 3. Participants

To be eligible for participation, laboratories either had to be operating on the national level or officially responsible for diagnosing H5 infections. A total of 149 laboratories from six WHO regions: Africa (AFR), Americas (AMR), Eastern Mediterranean (EMR), Europe (EUR), South-East Asia (SEAR), and Western Pacific (WPR), fulfilled the criteria and were invited to participate in this programme (Table 2). It included 118 of 128 National Influenza Centers listed on WHO web site (30 September 2009, date last accessed: <http://www.who.int/csr/disease/influenza/centres/en/>) and 31 other laboratories.

Table 2. Response of invited laboratories

Region	No. of laboratories				
	invited	responded	participated	received QAP samples	reported results
AFR	21	21	20	20	19
AMR	27	26	23	23	22
EMR	12	10	10	10	9
EUR	59	56	54	54	52
SEAR	8	8	6	6	6
WPR	22	22	22	22	21
Total	149	143	135	135	129

Of the 143 laboratories responded, eight laboratories did not participate in the EQAP due to lack of PCR capacity, lack of PCR reagents, lack of adequate training or other laboratory issues. All 135 laboratories were able to receive the shipment.

Of the 135 laboratories that received the shipment, 129 (96%) laboratories reported results within the specified time period or before the closing date for data collection, the results for one laboratory were excluded for analysis due to prolonged shipment time. The remaining six laboratories did not return their results.

A detailed list of the laboratories invited to participate in this EQAP and their responses are shown in Appendix 1.

## 4. Summary of results

### 4.1. Molecular amplification/detection testing results

The performance of individual participating laboratory was assessed based on the previous criteria. In brief, the qualitative results (that is, positive or negative) of the target genes tested were required; quantitative results (threshold cycle value,  $C_T$ ) were used only for reference. The performance of individual laboratories was assessed by adding up the number of correct results. No grades or ranking were assigned. The following were used when assessing laboratories' results:

- (i) failing to detect H5 samples and/or reporting the results as non-H5 subtype were recorded as an incorrect response;
- (ii) failing to detect H1 samples and/or reporting the results as non-H1 subtype were recorded as an incorrect response;
- (iii) failing to detect H3 samples and/or reporting the results as non-H3 subtype were recorded as an incorrect response;
- (iv) failing to report correct influenza A test results for H1/H3 samples if H1/H3 subtypings was not performed was recorded as an incorrect response;
- (v) failing to detect H1v samples and/or reporting the results as non-H1v subtype were recorded as an incorrect response;
- (vi) failing to report non-H1 and non-H3 test results for H1v samples if H1v subtypings was not performed was recorded as an incorrect response;
- (vii) reporting positive results for a sample that did not contain any viral RNA was recorded as an incorrect response;
- (viii) reporting 'H1v and H5' for H5 samples was recorded as correct response if using 'CDC real-time RTPCR Swine H1 Assay' (see comments).

A total of 101 participants returned correct results for all 10 samples (Table 3). An additional 12 participants returned correct results for nine samples, six participants eight samples, four participants seven samples and five participants returned fewer than seven correct results.

Table 3. Performance of the participants

Number of correct results (Max. possible = 10)	No. (%) of participants (N = 128)
10	101 (79)
9	12 (9)
8	6 (5)
7	4 (3)
5	3 (2)
4	2 (2)

Table 4. Sample characteristics and returned results

Copies/ $\mu$ L	Influenza A subtype	Clade	Sample No.	No. (%) of participants with correct results (N = 128)
4.727 x 10 <sup>2</sup>	H5N1	2.1	2009-19	119 (93)
3.338 x 10 <sup>2</sup>	H5N1	2.1	2009-17	114 (89)
1.867 x 10 <sup>4</sup>	H5N1	2.2	2009-20	122 (95)
1.690 x 10 <sup>3</sup>	H5N1	2.2	2009-14	119 (93)
1.970 x 10 <sup>3</sup>	H5N1	2.3.4	2009-15	125 (98)
1.176 x 10 <sup>3</sup>	H5N1	2.3.4	2009-11	117 (91)
7.933 x 10 <sup>3</sup>	H1N1	A/Brisbane/59/2007-like	2009-13	127 (99)
2.440 x 10 <sup>3</sup>	H3N2	A/Brisbane/10/2007-like	2009-18	120 (94)
2.037 x 10 <sup>2</sup>	H1N1v	A/California/4/2009-like	2009-12	126 (98)
4.560 x 10 <sup>1</sup>	H1N1v	A/California/4/2009-like	2009-16	128 (100)

The clade 2.3.4 H5 strain in two samples with similar concentrations, 2009-15 and 2009-11, were correctly reported by 125 (98%) and 117 (91%) participants respectively; while clade 2.1 H5 strain in two samples with similar concentrations, 2009-19 and 2009-17, were correctly reported by 119 (93%) and 114 (89%) participants respectively. The overall performance of the 256 tests on clade 2.3.4 H5 samples and clade 2.1 H5 samples were 91% and 95% respectively. For the clade 2.2 H5 strain in two samples with different concentrations, the samples with higher concentration 2009-20 and samples with lower concentration 2009-14 were correctly reported by 122 (95%) and 119 (93%) participants respectively.

The H1 strain in 2009-13 and H3 strain in 2009-18 were correctly reported by 127 (99%) and 120 (94%) participants respectively.

As for the H1v strain in two samples with different concentrations, the samples with higher concentration 2009-12 and samples with lower concentration 2009-16 were correctly reported by 126 (98%) and 128 (100%) participants respectively.

## 4.2. Methods of detection

This panel consisted of influenza A viruses representing different genetic clades 2 influenza A(H5N1) viruses, A(H1N1) virus, A(H3N2) virus and A(H1N1)v virus. The number of target genes used to identify influenza viruses varied among participants. Participants were assessed as giving false-positive and false-negative results for each target gene as follows:

- (i) for influenza A genes, unless otherwise specified, the target should be able to detect all of the influenza A viruses, A/NS, A/NP, A/NPv [specifically detect swine-origin H1 influenza viruses including influenza A(H1N1)v virus], A/M, A/Mv [specifically detect influenza A(H1N1)v virus], false-negative results were assigned on the basis of failure to detect virus in the samples, while false-positive results were assigned on the basis of reporting positive results for samples not containing the targeted gene.
- (ii) for HA subtyping genes, A/H1v, A/H1, A/H3, A/H5, false-negative results were assigned on the basis of failure to detect influenza A(H1N1)v virus, influenza A(H1N1) virus, A(H3N2) virus and A(H5N1) virus respectively; while false-positive results were assigned on the basis of reporting non-H1v, non-H1, non-H3, non-H5 results for samples containing influenza A(H1N1)v virus, A(H1N1) virus, A(H3N2) virus and A(H5N1) virus respectively;
- (iii) for NA subtyping of N1 gene, false-negative results were not recorded since most of the assays did not specifically detect H1N1, H1N1v and H5N1 viruses, while false-positive results were assigned on the basis of reporting N1 results for samples containing influenza A(H3N2) virus;
- (iv) for NA subtyping of N2 gene, false-negative results were assigned on the basis of failure to detect A(H3N2) virus, while false-positive results were assigned on the basis of reporting N2 results for samples containing non-influenza A(H3N2) virus.

If a participant provided more than one set of results for a target gene, a correct result would be assigned if at least one set of the results was correct.

Reports from the participating laboratories revealed different target genes were used to subtype influenza A viruses. The target genes used by participants and the number of participants with false-positive and false-negative results are shown in Table 5.

Table 5. Target genes tested by participants

Target genes tested	No. (%) of participants (N = 128)		
	Number using the target gene	False-positive	False-negative
for influenza A/B screening:			
- A/NS	1 (1)	not applicable <sup>a</sup>	1
- A/NP	2 (2)	not applicable <sup>a</sup>	1
- A/NPv	23 (18)	19 <sup>b</sup>	1 <sup>c</sup>
- A/M	124 (97)	not applicable <sup>a</sup>	8 <sup>d</sup>
- A/M (seasonal H1, H3)	1 (1)	0	0
- A/Mv	1 (1)	not specified	0
- A/M (non-seasonal H1, H3)	1 (1)	0	1
- B/NS	1 (1)	not specified	not applicable <sup>a</sup>
- B/M	1 (1)	0	not applicable <sup>a</sup>
- B/unknown	2 (1)	0	not applicable <sup>a</sup>
for HA subtyping:			
- A/H1	122 (95)	2 <sup>e</sup>	1
- A/H1v	120 (94)	2 <sup>g</sup>	0
- A/H3	120 (94)	0	7
- A/H5	126 (98)	3 <sup>h</sup>	19
- A/H7	2 (2)	0	not applicable <sup>a</sup>
for NA subtyping:			
- A/N1	20 (16)	0	not classified <sup>i</sup>
- A/N2	13 (10)	0	1

<sup>a</sup> All of the ten samples were influenza A viruses, either subtype H1, H1v, H3 or H5.

<sup>b</sup> 3/23 participants did not provide the test results of H5 samples, 19/20 reported positive H1v results for at least one of the six H5 samples, 3/19 reported the final results of H5 samples as 'H1v and H5'.

<sup>c</sup> 1/23 participants did not provide the test results for H1v samples.

<sup>d</sup> 3/124 participants did not provide the test results of the 10 samples.

<sup>e</sup> 1 participant reported H1 positive results for H1v samples and 1 participant reported H1 positive result for H3 sample.

<sup>g</sup> 2 participants reported H1v positive result(s) for at least one of the six H5 samples.

<sup>h</sup> 2 participants reported H5 positive result for H1v sample and 1 participant reported H5 positive result for H1 sample.

<sup>i</sup> Most of the assays for NA detection did not specify whether specific for subtype H1N1, H1N1v or H5N1.

For type A influenza viruses detection, the target most often used was the M gene (124/128, 97% participants), followed by NP gene (2/128, 2% participants) and NS gene (1/128, 1% participants). False negative results were reported by eight, one and one participants for these three target genes respectively. It is noted that some participating labs used different gene targets to differentiate seasonal from non-seasonal influenza virus: 23 (18%) participants reported using NPv gene to specifically detect swine influenza A viruses, 1 (1%) participant reported using Mv gene to specifically detect influenza A(H1N1)v virus, 1 (1%) participant reported using M gene to specifically detect influenza A(H1N1) and A(H3N2) viruses, 1 (1%) participant reported using M gene to specifically detect non-influenza A(H1N1) and A(H3N2) viruses. False-positive results were reported by 19 participants using NPv gene while false-negative results were reported by one participant using NPv gene and one participant using M gene to specifically detect non-influenza A(H1N1) and A(H3N2) viruses.

Four (3%) participants performed PCR for the detection of influenza type B virus and none of the participants gave false-positive results. As no sample in this dispatch contained influenza type B virus, there was no false-negative result.

For HA subtyping, all but two participants performed H5 subtyping and more than 90% of the participants reported the use of H1, H1v and H3 genes target to subtype influenza type A viruses. The proportion of false negative results was the highest among H5 gene followed by H3 gene and H1 gene, none for H1v gene. False positive results were recorded for H1 gene, H1v gene and H5 gene.

For NA subtyping, N1 and N2 genes were used by 20 (16%), 13 (10%) participants respectively. Among the 20 participants that performed N1 subtyping, 16 participants provided completed test results with eight participants using more than one set of primers/probes (Appendix 2). For N2 subtyping, false-negative results were reported by one participant, none of the participants gave false positive results.

The nucleic acid amplification tests used were mainly developed in-house. Commercial kits were only used rarely for the H1, H1v, H3, H7, N1 and N2 genes (one to four datasets), while for the detection of H5 and A/M genes, 10 out of 154 (6%) and 8 out of 140 (6%) datasets were generated through the use of commercial kits (Appendix 3-4). For in-house tests, except for the A/NP and H7 genes, the primers/probes were most commonly adapted from other researchers. Details on sources of the primers/probes are shown in Appendix 5-7.

Detection methods were based on either conventional or real-time detection. Conventional detection was most often used for the detection of A/NS, A/NP and B/NS genes, while real-time detection was most often used for A/NPv, A/M, B/M, H1, H3, H5, H7 and N1 genes. Both conventional and real-time detection were equally used for the detection of N2 gene (Appendix 3-4).

### 4.3. Category of testing errors

The results of this EQAP dispatch showed 79% participants returned all correct results. Incorrect results were most likely due to lack of specificity or sensitivity. The number of false negative results was higher than the number of false positive results (Table 5). Of the 27 laboratories with incorrect results, 23 reported incorrect results for H5 subtyping (i.e. false-positive, false negative or incorrect subtype results), three reported incorrect results for H1 subtyping and, four reported incorrect results for H1v subtyping and eight reported incorrect results for H3 subtyping (Table 6).

Table 6. Category of testing errors

Results	No. (%) of participants (N = 128)
All correct	101 (79)
Incorrect results for the following subtypes	
- H3	3 (2)
- H1, H3	1 (1)
- H5	15 (12)
- H5, H1	1 (1)
- H5, H1v	3 (2)
- H5, H3	3 (2)
- H5, H1, H1v, H3	1 (1)

#### 4.4. Quantification

Evaluation of quantification data have been attempted for Panel 6 and Appendix 9 summarizes the quantitative results for the three most common targets used for each virus containing sample.

Quantitative results for the three pairs of influenza A(H5N1) viruses from three genetic clades (clade 1, clade 2.3.2, clade 2.3.4) and one pair of influenza A(H1N1)v virus in this panel were analyzed. For A/M gene, the mean difference of  $C_T$  values between the paired samples were highest for H1N1v samples while for A/H5 gene, the mean difference of  $C_T$  values between the paired samples were highest for the H5 clade 2.3.4 samples.

Table 7. Analysis of quantification results of the paired samples for A/M and A/HA genes

Influenza A	A/M gene (include all of the targets)			A/HA gene		
	N <sup>a</sup>	mean diff	95% CI <sup>b</sup>	N	mean diff	95% CI
H5N1 clade						
- Clade 2.1 <sup>c</sup>	111	1.40	0.91 to 1.85	108	2.61	2.20 to 3.01
- Clade 2.2 <sup>d</sup>	112	2.32	1.91 to 2.73	117	2.56	2.14 to 2.98
- Clade 2.3.4 <sup>e</sup>	110	2.92	2.55 to 3.29	115	3.60	3.24 to 3.96
H1N1v <sup>f</sup>						
	114	3.34	2.90 to 3.77	111	3.04	2.68 to 3.40

<sup>a</sup> N, the number of paired datasets; mean diff, mean difference; CI, confidence interval.

<sup>b</sup> The 95% confidence interval was calculated by Paired *t* test.

<sup>c</sup> The mean difference was calculated by  $C_T$  of sample 2009-17 minus  $C_T$  of sample 2009-19.

<sup>d</sup> The mean difference was calculated by  $C_T$  of sample 2009-14 minus  $C_T$  of sample 2009-20.

<sup>e</sup> The mean difference was calculated by  $C_T$  of sample 2009-11 minus  $C_T$  of sample 2009-15.

<sup>f</sup> The mean difference was calculated by  $C_T$  of sample 2009-16 minus  $C_T$  of sample 2009-12.

#### 4.5. Correlation of correct results with interval from dispatch to receipt of panel

Of 128 laboratories reported the date of arrival, 57.8% participants received the panel within three days after dispatch. An additional 36 (28%) participants received the panel between four to seven days after dispatch, 13 (10%) received the panel at eight to 11 days interval while five (4%) participants received the panel longer than 11 days after dispatch (Table 8).

There is no significant difference in the number of participants with all correct results that received samples within first seven days and that received samples between eight to more than 11 days (Yates-corrected chi-square test;  $p = 0.289$ ). This is consistent with study conducted before dispatch to establish the stability of dried RNA after seven days storage of viral RNA at 37°C.

Table 8. Interval from dispatch to receipt of panel and number of correct results by participating laboratories

Days	No. of participants with number of correct results						Total
	4	5	7	8	9	10	
0-3	1	2	1	3	7	60	74
4-7	1	1	2	1	2	29	36
8-11			1	1	2	9	13
>11				1	1	3	5
Total	2	3	4	6	12	101	128

#### 4.6. Correlation of correct results with test turn-around time

EQAP samples should be handled, tested and performed in a similar manner as clinical samples. From the information provided by participants, test turn-around time (TAT) in individual laboratory was calculated and compared.

Of the 128 participants who returned result form, 126 provided both dates when testing was started and completed (analytic time); 126 provided both dates when testing was completed and result reported (post-analytic time); 128 provided dates when laboratory received the panel and result was reported (TAT).

The analytic time, post-analytic time and turn-around time reported by participants are shown in Table 9. For the analytic time and post-analytic time, most were between 0 to 7 days while for the TAT, most were longer than 28 days.

Table 9. Performance of participants and analytic, post-analytic and turn-around time required

Performance of participants		Time (days) required and no. of participants				
		0-7	8-14	15-21	22-28	>28
Analytic time						
- All	(N = 126)	92	18	10	4	2
- All correct results	(N = 101)	82	10	4	4	1
- All correct results for H5	(N = 105)	82	13	5	4	1
Post-analytic time						
- All	(N = 126)	114	7	2	1	2
- All correct results	(N = 101)	93	4	2	1	1
- All correct results for H5	(N = 105)	97	4	2	1	1
Turn-around time						
- All	(N = 128)	23	21	19	33	32
- All correct results	(N = 101)	22	19	17	24	19
- All correct results for H5	(N = 105)	22	19	17	27	20

When correlating performance with TAT, laboratory with TAT longer than 28 days showed significantly poorer H5 performance when compared to laboratory with TAT shorter than 28 days (Yates-corrected chi-square test;  $p = 0.006$  for all correct results;  $p = 0.004$  for correct results for H5).

#### 4.7. Significant factors affecting performance

As with previous panels, the performances of laboratories were analyzed according to the test methods used. In addition to assessing performance of in-house methods versus commercial kits and the use of conventional detection versus real-time detection; the number of H5 assays was also assessed in this panel.

Laboratory using real-time PCR in at least one test for the detection of H5 gene showed significantly better overall performance when compared to laboratory using conventional PCR ( $p = 0.006$ ). The use of commercial kit in at least one test for the detection of H5 gene and the use of second H5 assay as supplementary test, did not have a significant effect on the participants' overall performance (Table 10).

Table 10. Factors influencing the performance of laboratories for the detection of H5 gene<sup>a</sup>

Technical factor	Group									P
	All			A			B			
	N	n	%	N	n	%	N	n	%	
- real-time PCR assay	123	104	85	103	92	89	20	12	60	0.006
- commercial kit	124	10	8	104	6	6	20	4	20	0.110
- use of second H5 assay	123	30	24	103	28	27	20	2	10	0.164

<sup>a</sup> N, the number of laboratories responding to each specific question related to the listed technical factor.

n, the number of laboratory with positive answer to that specific question.

P, the *P* value for positive influence on all correct results calculated by Yates-corrected chi-square test.

A, group A represented participants without H5 incorrect results.

B, group B represented participants with H5 incorrect results.

## 5. Comments

The influenza A(H1N1)v virus was earlier detected by combined positive results for influenza A virus but negative for influenza subtypes H1N1, H3N2 and H5N1. Accurate diagnosis is critical to guide public health actions. On April 28, 2009, the real-time RT-PCR protocol for the detection and characterization of influenza A(H1N1)v virus was provided by CDC and was available from the WHO web site. This protocol provides three targets for detection and characterization of swine influenza: (1) A/M gene for universal detection of type A influenza viruses, (2) A/NPv gene for specific detection of type A swine-origin influenza A viruses including influenza A(H1N1)v virus and (3) A/H1v gene for specific detection of influenza A(H1N1)v virus.

Although influenza A(H1N1)v virus was first included in the present panel, results indicated that 124/128 (97%) participants are capable of correctly detecting influenza A/H1v in the samples by using at least one of the following targets: (1) A/H1v gene specific for influenza A(H1N1)v virus, (2) A/M gene specific for influenza A(H1N1)v virus and (3) A/HA gene specific for influenza A H1/H3. Although at least 22 different references were cited and used for A/HA specific gene to detect influenza A(H1N1)v virus, 91/128 (71%) participants used the CDC protocol. Among those 91 participants using the A/H1v gene to detect H1v samples, 19 participants also used A/NPv gene to screen for type A swine-origin influenza A viruses and reported positive results in at least one of the six H5 samples. However, 16 out of 19 (84%) participants were able to recognize the cross reactivity problems and interpreted the overall test results correctly as H5. The alignment of CDC primers/probe to NP gene of H1v and H5 samples were shown in Figure 1. Only 3 participants reported the results incorrectly as 'H1v and H5'. As the cross reactivity was not anticipated, the three participants were still categorized as giving correct response based on the correct individual H1v and H5 genes test results.

By correlating laboratory practice and results reported, factors that affect performance were found to be consistent with previous panels: (1) laboratories using real-time PCR in at least one test for the detection of H5 gene have better overall performance when compared to laboratory using agarose gel electrophoresis, (2) laboratories with test turn around-time (TAT) <28 days showed better performance when compared to laboratory with laboratory with test TAT >28 days.

The results of this Panel 6 provide a follow-up on the laboratory proficiency in influenza A diagnosis. Although the proportion of participants capable of correctly detecting all samples is still below 80%, this is the highest when compared with the previous five panels. The proportion of participants capable of correctly detecting all H5 samples, however, is the same as in Panel 5 (82%) and it may be related to the same low concentrations of RNA dispatched in Panel 5 and Panel 6. It is hoped that through participation in ongoing external quality assessments, the provision of high quality diagnostic services can be maintained.

**Appendix 1. Response of invited laboratories****AFR**

Country	No. invited	No. responded	No. joined	No. panel sent	No. reported
Algeria	1	1	1	1	1
Cameroon	1	1	1	1	1
Central African Republic	1	1	1	1	1
Côte d'Ivoire	1	1	1	1	1
Democratic Republic of Congo	1	1	1	1	1
Ethiopia	1	1	1	1	1
Gabon	1	1 <sup>c</sup>			
Ghana	1	1	1	1	1
Kenya	2	2	2	2	2
Madagascar	1	1	1	1	1
Mauritius	1	1	1	1	
Niger	1	1	1	1	1
Nigeria	2	2	2	2	2
Rwanda	1	1	1	1	1
Sénégal	1	1	1	1	1
South Africa	1	1	1	1	1
Tanzania	1	1	1	1	1
Uganda	1	1	1	1	1
Zambia	1	1	1	1	1
Total	21	21	20	20	19

**AMR**

Country	No. invited	No. responded	No. joined	No. panel sent	No. reported
Argentina	3	3	3	3	3
Brazil	3	3	3	3	2
Canada	1	1	1	1	1
Chile	1	1	1	1	1
Colombia	1	1	1	1	1
Costa Rica	1	1	1	1	1
Cuba	1	1	1	1	1
Ecuador	1	1	1	1	1
El Salvador	1	1	1	1	1
French Guiana	1	1	1	1	1
Guatemala	1				
Honduras	1	1 <sup>b</sup>			
Jamaica	1	1 <sup>b</sup>			
Mexico	1	1 <sup>c</sup>			
Panama	1	1	1	1	1
Paraguay	1	1	1	1	1
Peru	1	1	1	1	1
Trinidad and Tobago	1	1	1	1	1
United States of America	3	3	3	3	3
Uruguay	1	1	1	1	1
Venezuela	1	1	1	1	1
Total	27	26	23	23	22

**Footnotes:**

<sup>a</sup> The participant cannot be contacted due to invalid contact address.

<sup>b</sup> The participant did not join the Panel 6 due to lack of PCR capacity/PCR reagents/adequate training.

<sup>c</sup> The participant did not join the Panel 6 due to laboratory issues.

<sup>d</sup> The results of one participant were not included in the analysis due to long shipment time.

*Continued on following page*

## Continued-Appendix 1

**EMR**

Country	No. invited	No. responded	No. joined	No. panel sent	No. reported
Afghanistan	1	1	1	1	1
Egypt	2	2	2	2	2
Iran	1	1	1	1	1
Kuwait	1 <sup>a</sup>				
Lebanon	1 <sup>a</sup>				
Morocco	1	1	1	1	1
Oman	1	1	1	1	1
Pakistan	1	1	1	1	1
Sudan	1	1	1	1	1
Syrian Arab Republic	1	1	1	1	
Tunisia	1	1	1	1	1
Total	12	10	10	10	9

**EUR**

Country	No. invited	No. responded	No. joined	No. panel sent	No. reported
Albania	1	1 <sup>b</sup>			
Austria	1	1	1	1	1
Azerbaijan	1	1	1	1	1
Belarus	1	1	1	1	
Belgium	1	1	1	1	1
Bosnia and Herzegovina	2	1	1	1	1
Bulgaria	1	1	1	1	1
Croatia	1	1	1	1	1
Czech Republic	1	1	1	1	1
Denmark	1	1	1	1	1
Estonia	1	1	1	1	1
Finland	1	1	1	1	1
France	2	2	2	2	2
Georgia	1	1	1	1	1
Germany	1	1	1	1	1
Greece	2	2	2	2	2
Hungary	1	1	1	1	1
Iceland	1	1	1	1	1
Ireland	1	1	1	1	1
Israel	2	2	2	2	2
Italy	1	1	1	1	1
Kazakhstan	1	1	1	1	
Kyrgyzstan	1	1	1	1	1
Latvia	1	1	1	1	1
Lithuania	1	1	1	1	1
Luxembourg	1	1	1	1	1
Moldova	1	1	1	1	1
Montenegro	1				
Netherlands	2	2	2	2	2
Norway	1	1	1	1	1
Poland	1	1	1	1	1
Portugal	1	1	1	1	1
Republic of Macedonia	1	1	1	1	1
Republic of Serbia	2 <sup>a</sup>	1	1	1	1
Romania	2	2	2	2	2
Russia	3	3	3	3	3 <sup>d</sup>
Slovakia	1	1	1	1	1
Slovenia	1	1	1	1	1
Spain	3	3	3	3	3
Sweden	1	1	1	1	1
Switzerland	1	1	1	1	1
Turkey	2	2	2	2	2
Ukraine	1	1 <sup>b</sup>			
United Kingdom	3	3	3	3	3
Uzbekistan	1	1	1	1	1
Total	59	56	54	54	52

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## Continued-Appendix 1

**SEAR**

<b>Country</b>	<b>No. invited</b>	<b>No. responded</b>	<b>No. joined</b>	<b>No. panel sent</b>	<b>No. reported</b>
Bangladesh	1	1	1	1	1
Bhutan	1	1 <sup>b</sup>			
DPR Korea	1	1 <sup>b</sup>			
India	1	1	1	1	1
Indonesia	1	1	1	1	1
Myanmar	1	1	1	1	1
Sri Lanka	1	1	1	1	1
Thailand	1	1	1	1	1
Total	8	8	6	6	6

**WPR**

<b>Country</b>	<b>No. invited</b>	<b>No. responded</b>	<b>No. joined</b>	<b>No. panel sent</b>	<b>No. reported</b>
Australia	3	3	3	3	3
Cambodia	1	1	1	1	1
China	1	1	1	1	1
Fiji	1	1	1	1	1
French Polynesia	1	1	1	1	
Lao	1	1	1	1	1
Malaysia	2	2	2	2	2
Mongolia	1	1	1	1	1
New Caledonia	1	1	1	1	1
New Zealand	2	2	2	2	2
Papua New Guinea	1	1	1	1	1
Philippines	1	1	1	1	1
Republic of Korea	1	1	1	1	1
Singapore	1	1	1	1	1
Viet Nam	4	4	4	4	4
Total	22	22	22	22	21

Appendix 2. The 25 N1 subtyping datasets provided by 16 participants

No. of datasets generated using primer/probes for NA subtyping	Sample No.									
	H5N1						H1N1v		H1N1	H3N2
	Clade 1		Clade 2.3.2		Clade 2.3.4		2009-12	2009-16	2009-13	2009-18
	2009-19	2009-17	2009-20	2009-14	2009-15	2009-11				
5	+	+	+	+	+	+	-	-	-	-
5	-	-	-	-	-	-	+	+	-	-
3	-	-	-	-	-	-	-	-	+	-
1	-	-	-	-	-	-	+	+	+	-
1	+	+	+	+	+	+	+	+	+	-
2	+	-	+	-	+	-	+	+	+	-
1	+	-	+	+	+	+	+	+	+	-
1	-	-	+	-	-	-	-	-	+	-
1	-	-	+	+	+	+	-	-	+	-
1	-	-	-	-	-	-	-	+	+	-
1	+	-	+	+	+	+	+	-	-	-
1	+	-	+	+	-	-	-	-	-	-
1	+	-	+	+	+	+	-	-	+	-
1	+	-	+	+	+	-	-	-	-	-

Shadings denote dataset gave 100% correct results for N1 subtypes.

### Appendix 3. Procedures used for influenza A and influenza B screening

Procedures	The number of datasets <sup>a</sup>						
	A/NS N = 1	A/NP N = 2	A/NPv N = 23	A/M N = 140	A/M N = 3	B/NS N = 1	B/M N = 1
Nucleic acid amplification test							
- Commercial PCR				8			
- In-house PCR	1	2	23	132	3	1	1
Method							
- Conventional	1	2	1	20		1	
- Real-time			22	119	3		1
- Luminex				1			
Primers/probes							
- Commercial kit				8			
- Other researchers	1	1	22	124	1	1	1
- Own designed		1	1	8	2		

<sup>a</sup> Only datasets providing information of nucleic acid amplification, method and primers/probes were shown.

### Appendix 4. Procedures used for HA and NA subtyping

Procedures	The number of datasets <sup>a</sup>						
	H1 <sup>a</sup> N = 123	H1v <sup>a</sup> N = 130	H3 <sup>a</sup> N = 123	H5 <sup>a</sup> N = 154	H7 N = 2	N1 N = 29	N2 N = 12
Nucleic acid amplification test							
- Commercial PCR	0/3	0/4	0/2	4/10	1	3	1
- In-house PCR	1/120	3/126	7/121	24/144	1	26	11
Method							
- Conventional	1/29	3/12	5/28	12/30		9	6
- Real-time	0/93	0/118	2/94	16/123	2	20	6
- Luminex	0/1	0/0	0/1	0/1			
Primers/probes							
- Commercial kit	0/3	0/4	0/2	4/10	1	3	1
- Other researchers	0/107	3/114	6/105	24/134		15	8
- Own designed	1/13	0/12	1/16	0/10	1	11	3

<sup>a</sup> Only datasets providing information of nucleic acid amplification, method and primers/probes were shown.

<sup>b</sup> Number of datasets with incorrect results/Total number of datasets.

## Appendix 5. The number of datasets generated using different primers/probes from journal articles for influenza A/B screening, A/HA and A/NA subtyping

Source	A/M	A/H1 <sup>a</sup>	A/H1v <sup>a</sup>	A/H3 <sup>a</sup>	A/H5 <sup>a</sup>	A/N1	Others
Am J Respir Crit Care Med 1999, 159: 1316-1322	1						
BMC Infect Dis 2006, 6: 87	1						
Diagn Microbiol Infect Dis 2005, 53: 335-337	1						
J Clin Lab Anal 2002, 16: 163-166		0/1		0/1		1	1 <sup>b</sup>
J Clin Microbiol 1995, 33: 1180-1184		0/1		1/1			
J Clin Microbiol 1998, 36: 2990-2995		0/1		0/1			
J Clin Microbiol 2000, 38: 1552-1558	1	0/1					
J Clin Microbiol 2000, 38: 4096-4101	2						
J Clin Microbiol 2001, 39: 134-138		0/1		0/1			
J Med Virol 2003, 69: 132-144							1 <sup>c</sup>
J Virol Methods 1992, 39: 1-13							1 <sup>d</sup>
J Virol Methods 2001, 97: 13-22		0/1		0/1		1	1 <sup>b</sup>
J Virol Methods 2004, 117: 103-112		0/1		0/1			
J Virol Methods 2006, 131: 143-147						1	
J Virol Methods 2008 152 25-31	1	0/1		0/1	0/1		
Vet Microbiol 2009, 135: 253-260						1	
Virology 2008, 5: 77							1 <sup>b</sup>

<sup>a</sup> Number of datasets with incorrect results/Total number of datasets.

<sup>b</sup> Target A/N2.

<sup>c</sup> Target A/NP.

<sup>d</sup> Target A/NS.

## Appendix 6. The number of datasets generated using different primers/probes from published protocols for influenza A/B screening, A/HA and A/NA subtyping

Protocols			A/M	A/H1 <sup>b</sup>	A/H1v <sup>b</sup>	A/H3 <sup>b</sup>	A/H5 <sup>b</sup>	A/N1	Others
CDC (2009) <sup>b</sup>			53		3/91				23 <sup>c</sup>
CHP (2009) <sup>d</sup>			5	0/6	0/3	0/6			
HPA <sup>e</sup> VSOP 25.0			2				2/3		
HPA VSOP 29.0					0/3				
HPA VSOP 40.0							0/1		
HPA VSOP 41.0							1/5		
HPA VSOP 46.0							0/2		
HPA VSOP 48.0								1	
HPA SOP V5411				0/1		0/2			
HPA SOP V5413				0/1		0/1			
HPA SOP/VIR/072a			1				0/1		
WHO (2002) <sup>f</sup>							1/1		
WHO (2005) <sup>g</sup>							0/3	1	
WHO (2007) <sup>h</sup> from CHP, Hong Kong SAR							1/6		
WHO (2007) from EMC, the Netherlands			2						
WHO (2007) from NIID, Japan			7				4/8		

<sup>a</sup> Number of datasets with incorrect results/Total number of datasets.

<sup>b</sup> CDC protocol of realtime RTPCR for influenza A (H1N1) ([http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCR\\_SwineH1Assay-2009\\_20090430.pdf](http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCR_SwineH1Assay-2009_20090430.pdf))

<sup>c</sup> Target A/NP detect swine H1 influenza viruses including influenza A(H1N1)v virus

<sup>d</sup> CHP molecular diagnostic protocols for the detection of human swine influenza virus type A (subtype H1) ([http://www.chp.gov.hk/files/pdf/CHP\\_Protocols\\_for\\_the\\_Detection\\_of\\_Human\\_Swine\\_Influenza.pdf](http://www.chp.gov.hk/files/pdf/CHP_Protocols_for_the_Detection_of_Human_Swine_Influenza.pdf))

<sup>e</sup> Health Protection Agency (HPA), National Standard Methods - Virology ([http://www.hpa-standardmethods.org.uk/pdf\\_sops.asp](http://www.hpa-standardmethods.org.uk/pdf_sops.asp))

<sup>f</sup> WHO manual on animal influenza diagnosis and surveillance (<http://www.who.int/csr/resources/publications/influenza/whocdscsmcs20025.pdf>)

<sup>g</sup> Recommended laboratory tests to identify avian influenza A virus in specimens from humans ([http://www.who.int/csr/disease/avian\\_influenza/guidelines/avian\\_labtests2.pdf](http://www.who.int/csr/disease/avian_influenza/guidelines/avian_labtests2.pdf))

<sup>h</sup> Recommendations and laboratory procedures for detection of avian influenza A (H5N1) virus in specimens from suspected human cases ([http://www.who.int/csr/disease/avian\\_influenza/guidelines/RecAlllabtestsAug07.pdf](http://www.who.int/csr/disease/avian_influenza/guidelines/RecAlllabtestsAug07.pdf))

## Appendix 7. The number of datasets generated using different primers/probes from other institutions for influenza A/B screening, A/HA and A/NA subtyping

Influenza laboratory <sup>a</sup>	City	Country	A/M	A/H1 <sup>b</sup>	A/H1v <sup>b</sup>	A/H3 <sup>b</sup>	A/H5 <sup>b</sup>	A/N1	Others
WHO Collaborating Centre for Reference and Research on Influenza	Melbourne	Australia	1	0/1		0/1	1/1	2	
Pathcentre, Hospital Ave	Nedlands	Australia		0/1	0/1	0/1	0/1		
Virus Laboratory, Victorian Infectious Diseases Reference Laboratory	Victoria	Australia	1	0/1	0/1	0/1	0/1		
Institute of Clinical Pathology and Medical Research (ICPMR)	Wentworthville	Australia		0/1	0/1	0/1	0/1		
Public Health Agency of Canada	Winnipeg	Canda			0/1				
Virology Institute, Chinese CDC	Beijing	China	1	0/1	0/1	1/1			
The University of Hong Kong	Hong Kong SAR	China			0/1		0/1		
Tropical medicine Institute "Pedro Kouri"	Habana	Cuba			0/1				
Statens Serum Institut	Copenhagen	Demark	1	0/1	0/1		0/1		
U.S. Naval Medical Research Unit No.3	Cairo	Egypt					0/1		
National Institute for Health and Welfare	Helsinki	Finland			0/1				
Centre National de Référence de la Grippe Région Sud	Lyon	France	2						
Institut Pasteur, Paris, France	Paris	France	3	0/6	0/6	1/5	0/5	6	3 <sup>c</sup>
Robert Koch-Institute	Berlin	Germany	3	0/1	0/4	0/1	0/1	2	1 <sup>c</sup>
Influenza Surveillance Unit, National Virus Reference Laboratory	Dublin 4	Ireland	1 <sup>d</sup>						
Chaim Sheba Medical Center	Tel Hashomer	Israel	1			0/1			
Istituto Superiore di Sanità Centro Nazionale O.M.S. per l'Influenza	Roma	Italy		0/2		0/2			
Niigata University	Niigata	Japan					1/1		
National Institute of Infectious Diseases	Tokyo	Japan	2	0/1	0/1	0/2			
National Institute of Public Health and the Environment (RIVM)	Bilthoven	Netherlands	1	0/3	0/1	0/3	0/3	5	2 <sup>c</sup>
Erasmus Medical Centre (EMC)	Rotterdam	Netherlands	5	0/7	0/1	0/7	0/3	1	1

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## Continued-Appendix 7

Influenza laboratory <sup>a</sup>	City	Country	A/M	A/H1 <sup>b</sup>	A/H1v <sup>b</sup>	A/H3 <sup>b</sup>	A/H5 <sup>b</sup>	A/N1	Others
National Veterinary Institute	Oslo	Norway					0/1		
Norwegian Institute of Public Health	Oslo	Norway	1	0/1		0/1	0/1		
National Veterinary Research Institute (NVRI)	Pulawy	Poland					0/1		
National Public Health Institute of Slovakia	Bratislava	Slovakia		0/1	0/1	0/1			
Centro Nacional de Microbiologia	Madrid	Spain		1/3		2/3	2/3	1	1 <sup>f</sup>
Swedish Institute for Infectious Disease Control	Solna	Sweden	1	0/1	0/2	0/1	0/1		
Hôpital Cantonal Universitaire de Genève	Geneva	Switzerland	2 <sup>g</sup>				0/1	1	
Gartnavel General Hospital	Glasgow	United Kingdom	1	0/1		0/1	0/1		
National Institute for Biological Standards and Control	London	United Kingdom		0/1					1 <sup>c</sup>
WHO Collaborating Centre for Reference and Research on Influenza	London	United Kingdom			0/1				
Centres for Disease Control and Prevention	Atlanta	USA	29	0/65		2/67	10/82	1	1 <sup>c</sup>
J. Craig Venter Institute	Rockville	USA				0/1		1	
Hospital for Tropical Diseases	Ho Chi Minh City	Viet Nam			0/1				

<sup>a</sup> Influenza laboratories are listed hereafter, ordered by country and city where they are located.

<sup>b</sup> Number of datasets with incorrect results/Total number of datasets.

<sup>c</sup> Target A/N2.

<sup>d</sup> Target A/M specific for influenza A viruses other than A(H1N1) and A(H3N2).

<sup>e</sup> Target A/H7.

<sup>f</sup> Target A/NP.

<sup>g</sup> Target A/M specific for influenza A (H1N1)v viruses for one dataset; influenza A(H1N1) and A(H3N2) for another one dataset.

## Appendix 8. The number of datasets generated using different primers/probes from commercial kit for influenza A/B screening, A/HA and A/NA subtyping

Commercial kit <sup>a</sup>	Manufacturer	A/M	A/H1 <sup>b</sup>	A/H1v <sup>b</sup>	A/H3 <sup>b</sup>	A/H5 <sup>b</sup>	A/N1	Others
TaqMan Influenza A/H5 Detection Kit v1.0	Applied Biosystems					1/1		
TaqMan Influenza A/H5/H7/N1 Detection Kit v2.0	Applied Biosystems					1/2	1	
NucliSENS EasyQ Influenza H5 and N1	Biomerieux					1/1		
Flu A/B ASR	Cepheid	1						
AmpliSens Influenza virus A H5/N1-FRT	Epidemiological Research Institute, Moscow					1/3	1	
AmpliSens Influenza virus A/B-FL	Epidemiological Research Institute, Moscow	1						
AmpliSens Influenza virus A-H1 swine	Epidemiological Research Institute, Moscow			0/1				
AmpliSens Influenza virus A-H5/H7	Epidemiological Research Institute, Moscow							1 <sup>c</sup>
AmpliSens Influenza virus A-type-FL (subtypes H1N1 and H3N2)	Epidemiological Research Institute, Moscow						1	1 <sup>d</sup>
Influcheck kit (H1 and H3)	Euroclone	1	0/1		0/1			
Luminex's Respiratory Viral Panel (RVP) assay	Luminex	1	0/1		0/1	0/1		
INFLUENZA A/B Q - PCR Alert Kit	Nanogen Advanced Diagnost							
artus Influenza/H5 LC RT-PCR Kit	Qiagen					0/1		
RealTime ready Influenza A/H1N1 Detection Set	Roche	1	0/1					
RoboGene Detection of Bird Flu (BF) H5N1 RNA	Roboscreen					0/1		
Influenza virus A 1 Real Time RT-PCR Kit	Shanghai ZJ Bio-Tech Co. Ltd.		0/1					
LightMix for the detection of Influenza virus A H1 swine	TIB MOLBIOL		0/2					
LightMix for the detection of Influenza virus A M2	TIB MOLBIOL	2						
LightMix for the detection of Influenza virus A N1	TIB MOLBIOL							

<sup>a</sup> Commercial kits are listed hereafter, ordered by manufacturer.

<sup>b</sup> Number of datasets with incorrect results/Total number of datasets.

<sup>c</sup> Target A/H7.

<sup>d</sup> Target A/N2.

Appendix 9. Summary of quantitative results<sup>a</sup>

## Influenza A(H5N1) clade 2.1 samples

Influenza virus (type/target)	2009-19					2009-17				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
- A/M	113	25.41	3.20	15.93	39.83	111	26.84	3.03	19.23	40.10
- A/H5	114	30.03	3.71	21.16	41.00	110	32.43	3.65	23.34	42.84
- A/N1	8	30.21	3.41	24.98	33.06	6	33.40	2.29	31.20	36.00

## Influenza A(H5N1) clade 2.2 samples

Influenza virus (type/target)	2009-20					2009-14				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
- A/M	113	23.09	3.35	14.60	36.00	113	25.46	3.73	18.82	40.00
- A/H5	117	24.97	4.05	17.40	38.05	118	27.55	3.55	18.80	40.00
- A/N1	9	27.17	3.38	21.88	31.81	9	29.56	3.88	22.60	34.00

## Influenza A(H5N1) clade 2.3.4 samples

Influenza virus (type/target)	2009-15					2009-11				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
- A/M	111	23.14	3.54	15.76	39.00	111	26.01	3.90	13.35	43.00
- A/H5	118	26.71	4.10	16.92	39.54	115	30.02	3.68	21.35	43.00
- A/N1	9	29.20	3.42	25.18	35.00	8	33.54	3.37	30.20	39.00

## Influenza A seasonal (H1N1) and (H3N2) samples

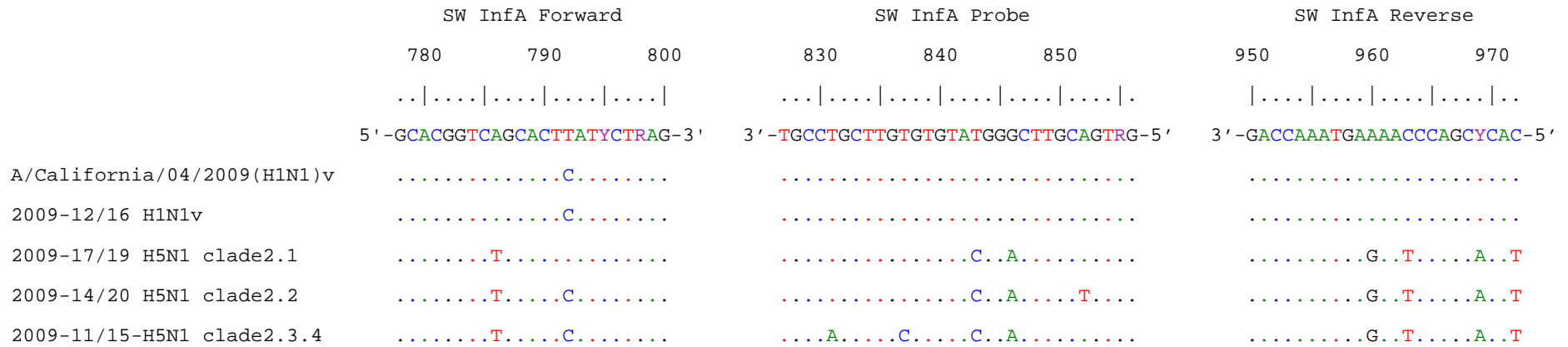
Influenza virus (type/target)	2009-13 (H1N1)					2009-18 (H3N2)				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
- A/M	111	24.60	2.89	16.09	38.00	111	26.49	3.54	11.14	39.00
- A/H1 or A/H3	93	26.57	3.68	15.06	39.60	92	28.97	3.81	21.15	43.73
- A/N1 or A/N2	7	28.98	2.28	24.90	31.62	6	30.65	4.41	22.28	34.10

## Influenza A(H1N1)v samples

Influenza virus (type/target)	2009-12					2009-16				
	N	Mean	SD	Min	Max	N	Mean	SD	Min	Max
- A/M	114	22.93	3.09	16.58	32.13	115	26.28	3.01	20.00	41.13
- A/H1v	112	25.97	3.74	19.00	39.04	112	29.00	3.11	21.00	38.74
- A/N1	6	24.64	2.78	22.10	29.00	6	28.09	2.33	25.10	31.00

<sup>a</sup> N, the number of datasets; mean, average; SD, standard deviations; Min, minimum value; Max, maximum value.

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Location of the oligonucleotides were based on NP gene of the strain A/California/4/2009 (H1N1)v, GenBank Accession: FJ966083.

Figure 1. The alignment of CDC primers/probe to NP gene of H1v and H5 samples.