

# Real-time RT-PCR assays for type and subtype detection of influenza A and B viruses

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Influenza viruses type A (H3N2 and H1N1 subtypes) and B are the most prevalently circulating human influenza viruses. However, an increase in several confirmed cases of high pathogenic H5N1 in humans has raised concerns of a potential pandemic underscoring the need for rapid, point of contact detection. In this report, we describe development and evaluation of 'type,' i.e., influenza virus A and B, and 'subtype,' i.e., H1, H3, and H5, specific, single-step/reaction vessel format, real-time RT-PCR assays using total RNA from archived reference strains, shell-vial cultured and uncultured primary (throat swab/nasal wash) clinical samples. The type A and B specific assays detected all 16 influenza type A viruses and both currently circulating influenza B

lineages (Yamagata and Victoria), respectively. 'Type' and 'subtype' specific assays utilize one common set of thermocycling conditions, are specific and highly sensitive (detection threshold of approximately 100 target template molecules). All clinical specimens and samples were evaluated using both the unconventional portable Ruggedized Advanced Pathogen Identification Device (RAPID) and standard laboratory bench LightCycler instruments. These potentially field-deployable assays could offer significant utility for rapid, point of care screening needs arising from a pandemic influenza outbreak.

**Keywords** H1, H3, H5, influenza A/B, RT-PCR.

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## Background

In humans, Influenza is an acute, highly contagious respiratory disease caused primarily by influenza viruses type A and B. The infectivity of influenza viruses is augmented considerably due to viral alteration of antibody targeted surface glycoproteins, i.e., 'drift,' giving rise to annually occurring epidemics. In contrast to annual epidemics, pandemics occur at 10–50 year intervals, arising by re-assortment, i.e., 'shift' between human and avian/porcine viruses.<sup>1</sup> Of significant concern are human infections arising from avian H5 influenza A virus considered by many as an emerging pandemic threat.<sup>2–4</sup>

Currently, several conventional 'gold standard' procedures for viral identification are well-established in the clinical reference laboratory.<sup>5–8</sup> However, these methods require 3–6 days for definitive diagnosis as well as propagation of live (and potentially infectious) influenza viral strains and are of very little 'point of care' value. Rapid ELISA-based 'flu/no flu' assays have been developed and are commercially available. Although fast and easily used,

these assays often exhibit specificity and sensitivity shortcomings and do not subtype influenza virus type A, e.g., H1, H3, and H5.<sup>9–12</sup>

Traditional RT-PCR has become an effective methodology for viral detection, typing, and subtyping.<sup>13–15</sup> Although RT-PCR exhibits high sensitivity and considerably shorter detection time than conventional virological methods, drawbacks, such as extraction complexity, post-PCR analysis, and increased potential for cross-contamination have been noted.<sup>16,17</sup> Several dual step, real-time (rRT-PCR) assays have appeared in the literature but require either nested PCR or use of random hexamers.<sup>18–20</sup> Likewise, single-step assays have been developed, but require a variety of reagents of differing concentrations as well as thermocycling parameters.<sup>21</sup> In 2004, the artus Influenza/H5 kit (Qiagen, Valencia, CA, USA) was made commercially available (for research use only), combining a broad screening capability for detecting type A (15 of 16 HA types) and type B viruses. In 2005, the Laboratory Response Network (LRN) single-step assay was developed and subsequently approved for use by the Food Drug

Administration (FDA) for detection of avian H5 influenza.<sup>22–24</sup> However, this assay does not detect nor distinguish commonly circulating strains, i.e., H1, H3, and influenza B viruses. More recently (2006), the TaqMan Influenza A/H5 virus detection kit (Applied Biosystems, Foster City, CA, USA) consisting of two assays; one designed to detect influenza type A (M gene target) and a second specific for the H5 subtype (hemagglutinin gene target), was made commercially available but for research use only. Although the artus Influenza/H5 kit offers a broader screening capability than that of the TaqMan Influenza A/H5 assay, neither of these reagent kits subtype specifically commonly circulating human strains. Recently, Di Trani and coworkers describe a sensitive, one-step, rRT-PCR method for detection of type A influenza viruses (13 of 16 HA types) using a minor groove binding probe;<sup>25</sup> however, the method of Di Trani does not differentiate type A and B viruses nor subtype the respective type A viruses.

## Objective

The objective of this work was twofold: (i) to develop and (ii) evaluate 'type,' i.e., influenza virus A and B, and 'subtype,' i.e., H1, H3, and H5, specific, rRT-PCR assays. Furthermore, the assays were to be carried out using one identical and common set of thermocycling conditions (single-step/reaction vessel format) and two rRT-PCR platforms; the unconventional 50 pound portable Ruggedized Advanced Pathogen Identification Device (RAPID) and standard laboratory bench LightCycler instrumentation.

## Patients/methods

### Collection of clinical samples and virology

All original primary specimens (throat swab/nasal washes) and cultured samples used in this study were collected over 7 (99/00, 00/01, 01/02, 02/03, 03/04, 04/05, and 05/06) and 3 (03/04; 04/05, and 05/06) influenza seasons, respectively under the auspices of the Department of Defense Global Emerging Infectious Surveillance (DoD-GEIS) network. Primary specimens were collected within the first 48–72 h of symptom onset from patients presenting with a fever  $\geq 100.5^{\circ}\text{F}$  oral and cough or sore throat. Dacron throat swab specimens were submerged in 3.0 ml viral transport media (M4 MicroTest Multi-Microbe Media, Remel, Lenexa, KS, USA). Submerged throat swabs and saline nasal wash material (5 ml) were shipped on dry ice to Brooks City Base, San Antonio, TX, USA. Propagation of influenza viruses from primary specimens was achieved using the centrifugation-enhanced Shell-Vial culture technique followed by typing using influenza virus A or B specific monoclonal antibodies (Chemicon International,

Temecula, CA, USA) per manufacturer's recommendations and fluorescent microscopy.<sup>26</sup> Aliquots (0.25 ml) of primary specimens (before and after culturing) served as source of specimen RNA.

### Extraction of RNA

RNA extraction was achieved using the Qiagen M48 automated bead-based extraction robot (Qiagen) with the Mag-Attract Virus Mini M48 kit (Qiagen) per manufacturer's protocols, eluted in 50  $\mu\text{l}$  of Elution Buffer and stored at  $-80^{\circ}\text{C}$  until used.

### Genomic primer/probe design

Primer/probe design was based upon sequence data obtained from the Los Alamos National Laboratory and Department of Defense data bases. Type specific (influenza A and B) assays target a highly conserved region of the matrix protein (MP) gene and were designed based on 100 and 50 alignments common to all 16 influenza A virus subtypes and both influenza B lineages (B/Victoria and B/Yamagata), respectively. H1, H3, and H5 influenza A subtype specific assays target conserved regions of the respective hemagglutinins (HA). H1 primer/probe sequence alignment was achieved using 51 geographically diverse strains obtained during the 2005/06 and 2006/07 influenza seasons including the A/New Caledonia/20/99 vaccine strain. H3 primer/probe sequence alignment was achieved using 140 H3N2 field strains collected between 2004 and 2006. H5 primer/probe sequence design was based upon alignment analysis of 22 H5N1 clinical isolates representing clades 1 and 2 (subclades 1, 2, and 3) viruses. All primers and probes were procured from Applied Biosystems.

### Real-time RT-PCR platforms

The laboratory-based Lightcycler 2.0 instrument (Roche Molecular Diagnostics, Indianapolis, IN, USA) and its lightweight portable (50 lbs) version, the Ruggedized Advanced Pathogen Identification Device (RAPID, Idaho Technologies, Salt Lake City, UT, USA) are both 32-well capillary, real-time instruments which employ similar components and operational software. The RAPID is configured within a hardened case and can be employed at the point of care.

### Real-time RT-PCR amplification

Primer and probe sequences are shown in Table 1. Primer pair melting points are within  $2^{\circ}\text{C}$  and anneal/extend at  $58\text{--}60^{\circ}\text{C}$ . The respective probes anneal/extend  $8\text{--}10^{\circ}\text{C}$  higher than that of the primers. Thermocycling operates in a rapid, two temperature format with annealing and extension (30 s) facilitated by the short nature of the respective amplicons. Due to genetic variability in the influenza viral genome, degenerate nucleotides were placed at specific loci.

**Table 1.** Primer/probe sequences\* used for rRT-PCR amplification and *in vitro* generation of cRNA target templates

Primer	Sequence	Gene target-nucleotide position*	Amplicon (bp)
<i>Real-time RT-PCR</i>			
Type A		Matrix (Segment 7)	195
Forward	taaccgaggtcgaacgta	36 → 54	
Reverse	gcacggtgagcgtgaa	215 → 230	
Probe	(FAM)-tcaggccccctcaaagc	74 → 90	
Type B		Matrix (Segment 7)	119
Forward	ggaattgcaaaggatgtaatggaa	673 → 696	
Reverse	agaacaaattgaaagaatctgaaatggt	764 → 791	
Probe	(FAM)-atgggaaattcagctct	721 → 731	
Subtype H1		H1 Hemagglutinin (Segment 4)	89
Forward	agycctccttccagaatgtaca	948 → 970	
Reverse	agtctgtarccatycctaattttg	1012 → 1036	
Probe	(FAM)-tctccaaagtatgcagg	973 → 988	
Subtype H3		H3 Hemagglutinin (Segment 4)	113
Forward	aatacgaagtgggaaaagctca	777 → 798	
Reverse	gcccrtatgtgatycgtttac	889 → 911	
Probe	(FAM)-tgatcagatgcacccat	803 → 821	
Subtype H5		H5 Hemagglutinin (Segment 4)	144
Forward	actayccgcagtattcagaagaagc	1511 → 1535	
Reverse	gaccagcyaycatgattgcca	1634 → 1654	
Probe	(FAM)-agagrsgaataagtgg	1546 → 1562	
<i>cRNA target templates</i>			
Type A		Matrix (Segment 7)	1027
Forward**	gctaatacgcactactatagggagaagcaaaagcaggtagatatt	1 → 20	
Reverse	agtagaacaaggtagtttttac	1004 → 1027	
Type B		Matrix (Segment 7)	1061
Forward**	gctaatacgcactactatagggagatcgctgtttggagacacaat	7 → 26	
Reverse	ctccaaaactgtttcaccca	1048 → 1067	
Subtype H1		H1 Hemagglutinin (Segment 4)	1204
Forward**	gctaatacgcactactatagggagaagcaggggaaaaataaaa	7 → 23	
Reverse	gtaatcccgtaatcgca	1193 → 1210	
Subtype H3		H3 Hemagglutinin (Segment 4)	1192
Forward**	gctaatacgcactactatagggagaactatcattgctttgagc	7 → 24	
Reverse	atggctgcttgagtgctt	1181 → 1198	
Subtype H5		H5 Hemagglutinin (Segment 4)	1658
Forward**	gctaatacgcactactatagggagatcatctgcaaatggagaaaaat	4 → 22	
Reverse	aaggatagaccagctaccatga	1640 → 1661	

Genomic primer/probe design, rRT-PCR and *in vitro* generation of cRNA target templates were carried out as previously described under 'Patients/methods'

\*All sequences are listed 5' → 3'.

\*\*Inclusive of the 22 nucleotide T7 promoter sequence with the gc linker (5' end).

r = 50% mixture of a and g.

y = 50% mixture of c and t.

Real-time amplification was performed in a single-step/reaction vessel format. Using the UltraSense Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA), 2  $\mu$ l RNA was added to 18  $\mu$ l master mix containing the following components at the indicated final concentrations: 1X reaction buffer, 1X enzyme mixture containing 500 nM of each primer and 300 nM probe labeled at the 5' end with 6-carboxyfluorescein (FAM)

reporter dye and at the 3' end with a non-fluorescent quencher and minor groove binder. Thermocycling was carried out as follows: 30 min at 45°C and 2 min at 95°C for reverse transcription (RT) and denaturation, respectively, followed by 40 amplification cycles consisting of 95°C for 5 s (denaturation) and 60°C for 30 s (extension). Amplification efficiency was determined using the  $C_T$  slope method (cf. Fig. 1, frame B) according to the equation:

$E = [10^{(-1/\text{Slope})} - 1] \times 100$ . All assays described here exhibited greater than 98.5% amplification efficiency.

For each analysis, 'no template' and 'positive' controls were included. Baseline fluorescence for each analysis was manually adjusted to that of the respective 'no template' control reaction. The 'positive' control (0.1 ng cRNA) gives rise to an increase in fluorescence intensity relative to the no template baseline ( $C_T$  value between 18 and 22). A 'positive' unknown is defined as amplification exceeding baseline fluorescence with a corresponding  $C_T$  value not exceeding 36 in a 40 cycle run. All original, i.e., uncultured specimens and cultured samples reported here using both platforms exhibited  $C_T$  range values of 26–35 ( $n = 144$ , mean = 31.5) and 17–27 ( $n = 407$ , mean = 23), respectively.

### Generation of cRNA target templates

Reverse and forward primers for *in vitro* generation of target complementary RNA (cRNA) templates corresponding to Influenza type (A/B) and Influenza A subtype (H1, H3, and H5) RNA sequences are shown in Table 1.

Briefly, traditional RT-PCR was carried out as follows: 5  $\mu$ l viral RNA was added to a 45  $\mu$ l master mix containing the following components at the indicated final concentrations: 1X reaction buffer with 1.6 mM MgSO<sub>4</sub>, 1X enzyme mixture containing 400 nM HA or MP primer pairs using the SuperScript III One-Step RT-PCR System (Invitrogen). Reverse transcription was carried out at 50°C for 30 min followed by a 'hot start' step (2 min) at 95°C. Thermocycling (40 amplification cycles) was carried out as follows: 30 s at 95°C, 15 s at 52°C, 1 min at 68°C with final extension for 7 min at 68°C. PCR reaction product (5  $\mu$ l) was subjected to analytical electrophoresis on 2% pre-cast gels containing ethidium bromide (Invitrogen) and remaining product (45  $\mu$ l) purified using the Qiaquick PCR Purification Kit (Qiagen). *In vitro* transcription was carried out for 4 h using the T7 MegaScript Kit (Ambion, Austin, TX, USA) per manufacturer's recommendations. Reactions were subjected to nuclease digestion using Turbo DNase (Ambion) and subsequently purified using the MegaClear kit (Ambion). RNA was quantitated using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer, aliquoted and served as control cRNA.

### Nucleotide sequencing

Purified amplicons were cloned using a Topo 2.0 Cloning Kit (Invitrogen) and sequenced using the Big Dye Terminator v3.1 reagent Kit (Applied Biosystems). Unincorporated fluorescent nucleotides were removed using a Dye Ex 96-well plate kit per manufacturer's recommendations (Qiagen). Nucleotide sequencing was performed using an ABI 3100 Genetic Analyzer (ABI Inc.).

## Results

As shown in Table 2, influenza A and B virus type specific assays detected all known influenza A hemagglutinin sub-

**Table 2.** Detection of influenza virus type (A/B) strains

Subtype	Strain	Flu A Univ*	Flu B Univ*
H1N1	DK/NJ/7717-70/95	+	-
H1N1	CK/NY/21665-73/98	+	-
H2N2	CK/NY/3749-7/96	+	-
H2N4	DK/LA/8174/86	+	-
H3N8	ENV/NY/19019-6/98	+	-
H4N6	DK/CZECH/56	+	-
H4N8	DK/ALB/286/78	+	-
H5N1	TK/ENG/50-92/91	+	-
H5N1	CK/SCTT/59	+	-
H5N1	MA/OH/184/86	+	-
H5N1	A/SE Asia/2003	+	-
H5N2	CK/MA/11801/86	+	-
H5N2	CK/PUE/8624-602/94	+	-
H5N2	PH/NJ/1355/95	+	-
H5N3	DK/SING/97	+	-
H5N3	TK/CA/6878/79	+	-
H5N8	TK/IR/83	+	-
H5N9	RATITE/NY/12716/84	+	-
H6N2	CK/NY/14677-13/99	+	-
H7N1	SB/IL/35445-136/92	+	-
H7N1	TK/IT/4580/99	+	-
H7N2	CK/PA/13552-1/98	+	-
H7N2	EQ/NY/13142-5/94	+	-
H7N2	TK/PA/7975/97	+	-
H7N3	CK/PAK/13692/95	+	-
H7N3	MA/NETHERLANDS/12/00	+	-
H7N3	Q/AR/16309-2/94	+	-
H7N4	CK/NSW/1688/97	+	-
H7N7	CK/VIC/85	+	-
H7N7	EQ/PRAGUE/E5302/56	+	-
H8N4	TK/ONT/6188/67	+	-
H8N8	DK/VIC/8211-18-1400/92	+	-
H9N2	A/CK/HK/G9/97	+	-
H9N2	CK/KOREA/96006/96	+	-
H9N2	CK/NJ/12220/97	+	-
H10N7	TK/VA/31409/91	+	-
H10N7	CI/GER/N/49	+	-
H11N1	DK/ENG/56	+	-
H11N1	CK/NJ/15906-6/96	+	-
H12N5	DK/LA/18813/87	+	-
H12N5	DK/ALB/60/76	+	-
H13N6	GULL/6411/MD/704/77	+	-
H14N5	MA/GURJEV/263/82	+	-
H15N9	SW/AUSTR/2576/79	+	-
H16N3	A/BHG/SWEDEN/2/99	+	-
B/Yam	B/Arizona/135/2005	-	+
B/Vic	B/Arizona/140/2005	-	+
B/Vic	B/Nepal/1120/2005	-	+
B/Yam	B/Florida/925/2004	-	+

**Table 2.** (Continued)

Subtype	Strain	Flu A Univ*	Flu B Univ*
B/Vic	B/Hawaii/22/2004	–	+
B/Vic	B/Brisbane/32/2002	–	+

Influenza virus A and B detection by real-time RT-PCR was carried out as previously described under 'Patients/methods'.

Total RNA for influenza virus A H1-15 subtypes was obtained from Dr. David Suarez, Southeast Poultry Research Laboratory, USDA Agricultural Research Service, Athens, Georgia 30605. H16 RNA was provided by Drs. R.A. Fouchier and A.D. Osterhaus of the Department of Virology, Erasmus Medical Center, The Netherlands. Influenza B virus reference strains were obtained from the Department of Defense Global Emerging Infectious Surveillance (DoD-GEIS) network.

\*Determined by type A and B specific assays.

B/Yam = Type B Yamagata lineage; B/Vic = Type B Victoria lineage.

types (H1-H16) and both type B (Yamagata and Victoria) viruses, respectively. Importantly, no cross-reactivity was observed between influenza A and B virus specific probes supporting the very specific nature of these assays.

H1, H3, and H5 influenza A subtype specific assays were initially evaluated using 180 archived clinical isolates. As shown in Table 3, 178 samples were correctly typed and subtyped in research blinded fashion; the far greater number of samples identified as type A influenza (91%) with the remainder, i.e., 9% being type B influenza. Of the type A influenza samples, the H3 subtype was the most prevalent (93.2%) with the remaining 6.8% H1 subtype. Two samples (District of Columbia) tested influenza virus negative (using type and subtype assays reported here) and were later confirmed as being Coxsackie B and Adenovirus (data not shown). Consistent with no cross-reactivity of type specific probes, no subtype specific probe cross-reactivity, i.e., H1, H3, and H5 assay cross-reactivity was observed. Furthermore, 40 commonly encountered bacteria/viruses were tested concurrently and did not amplify using either type and subtype specific primer/probes listed in Table 1 (data not shown).

Typing and subtyping influenza viruses at the point of care using uncultured (low viral titer) primary specimens is crucial for expanded surveillance. Shown in Table 4 is analysis of 167 uncultured primary clinical samples. Of the 167 specimens, 100 were correctly identified, i.e., typed (Influenza A or B, 60 and 40, respectively) and type A samples subtyped H1 or H3, 12 (20%) and 48 (80%), respectively. Furthermore, 67 negative influenza samples were subsequently determined to be culture negative for influenza viruses (data not shown). Of the 60 influenza A samples, 16 initially tested influenza negative in contrast to

**Table 3.** Detection of influenza virus types (A/B) and subtypes (H1, H3, and H5) in cultured clinical isolates by rRT-PCR

Location (samples)	Flu A/B*	H1**	H3**	H5**	Flu A/B***
United States					
Alabama (2)	2/0	0	2	0	2/0
Alaska (4)	3/1	0	3	0	3/1
Arizona (9)	7/2	0	7	0	7/2
California (4)	4/0	0	4	0	4/0
Colorado (23)	22/1	0	22	0	22/1
Connecticut (1)	1/0	0	1	0	1/0
District of Columbia (4) <sup>†</sup>	1/1	0	1	0	1/1
Hawaii (11)	9/2	0	9	0	9/2
Illinois (4)	4/0	0	4	0	4/0
Maryland (6)	6/0	0	6	0	6/0
New Jersey (3)	3/0	0	3	0	3/0
New York (1)	1/0	0	1	0	1/0
Oklahoma (5)	5/0	0	5	0	5/0
Texas (24)	24/0	0	24	0	24/0
Virginia (2)	2/0	0	2	0	2/0
Country					
Ecuador (1)	1/0	0	1	0	1/0
England (7)	4/3	1	3	0	4/3
Guam (2)	2/0	1	1	0	2/0
Iraq (1)	1/0	0	1	0	1/0
Italy (1)	1/0	0	1	0	1/0
Japan (21)	19/2	1	18	0	19/2
Ketchikan (1)	1/0	0	1	0	1/0
Kenya (2)	1/1	1	0	0	1/1
Korea (12)	12/0	5	7	0	12/0
Kuwait (3)	2/1	1	1	0	2/1
Peru (21)	19/2	0	19	0	19/2
Qatar (4)	4/0	1	3	0	4/0
Turkey (1)	1/0	0	1	0	1/0
Total Samples = 180	162/16	11	151	0	162/16

Real-time RT-PCR analyses and traditional virus isolation/identification of shell vial cultured clinical samples were carried out as previously described under 'Patients/methods'

\*Determined by type A and B specific assays.

\*\*Determined by subtype (H1, H2, and H3) specific assays.

\*\*\*Determined by traditional 'gold standard' virus isolation/identification.

<sup>†</sup>Two of 4 District of Columbia samples tested negative for influenza virus using type/subtype specific assays reported here and were subsequently confirmed as being influenza negative by traditional 'gold standard' virus isolation/identification.

original culture data. The inability to detect 16 of the 100 influenza specimens could have arisen from base pair sequence 'drift' in the respective primer/probe binding regions, less than threshold amounts of extracted target RNA arising from prolonged storage/degradation at  $-80^{\circ}\text{C}$  and poor sample collection. Therefore, aliquots of all 16 samples were removed and transferred to monolayer primary monkey kidney (PMK) culture tubes and Shell Vials for further analysis. After 48 h, 10 of the 16 Shell Vial

**Table 4.** Detection of influenza virus types (A/B) and subtypes (H1, H3, and H5) in uncultured primary clinical specimens by rRT-PCR

Location (samples)	Flu A/B*	H1**	H3**	H5**	Flu A/B***
United States					
Alabama (1)	0/1	0	0	0	0/1
Arkansas (4)	4/0	3	1	0	4/0
California (1)	1/0	0	1	0	1/0
Delaware (1)	1/0	0	1	0	1/0
District of Columbia (2)	0/2	0	0	0	0/2
Georgia (2)	2/0	0	2	0	2/0
Hawaii (14) <sup>††(4)</sup>	14/0	1	13	0	14/0
Mississippi (1) <sup>††(1)</sup>	1/0	0	1	0	1/0
New Jersey (2)	2/0	1	1	0	2/0
New Mexico (2)	0/2	0	0	0	0/2
Oklahoma (1)	1/0	0	1	0	1/0
South Carolina (1)	1/0	0	1	0	1/0
Texas (3) <sup>†</sup>	1/1	1	0	0	1/1
Virginia (16)	1/15	0	1	0	1/15
Washington (1)	1/0	0	1	0	1/0
Country					
Guam (2) <sup>††(2)</sup>	2/0	0	2	0	2/0
Iraq (1)	1/0	0	1	0	1/0
Italy (2)	1/1	0	1	0	1/1
Japan (5) <sup>††(2)</sup>	3/2	2	1	0	3/2
Kenya (2)	1/1	0	1	0	1/1
Korea (5) <sup>††(2)</sup>	5/0	1	4	0	5/0
Kuwait (1)	1/0	1	0	0	1/0
Nepal (69) <sup>†, ††(1)</sup>	1/2	0	1	0	1/2
Okinawa (1)	1/0	0	1	0	1/0
Peru (8)	4/4	1	3	0	4/4
Qatar (2)	2/0	1	1	0	2/0
Thailand (13) <sup>††(2)</sup>	4/9	0	4	0	4/9
Turkey (2) <sup>††(2)</sup>	2/0	0	2	0	2/0
United Kingdom (2)	2/0	0	2	0	2/0
Total Samples = 167	60 <sup>††(16)</sup> /40	12	48	0	60/40

Real-time RT-PCR analyses and traditional virus isolation/identification of primary clinical specimens (throat swabs/nasal washes) were carried out as previously described under 'Patients/methods'

\*Determined by type A and B specific assays.

\*\*Determined by subtype (H1, H2, and H3) specific assays.

\*\*\*Determined by traditional 'gold standard' virus isolation/identification.

<sup>†</sup>One of 3 Texas samples and 66 of 69 Nepal samples tested negative for influenza virus using type/subtype specific assays reported here and were all subsequently confirmed as being influenza negative by traditional 'gold standard' virus isolation/identification.

<sup>††</sup>Primary uncultured samples requiring culturing for identification. The number of samples is indicated in the parenthesis.

enriched samples tested positive by type specific rRT-PCR and standard immunoreagent fluorescence staining. The remaining six samples were checked/screened daily thereafter and three of six tested positive 5 days post-inoculation and the remaining three testing positive 9 days post-inoculation. All 16 influenza type A amplicons were further validated by sequence analysis (data not shown). The overall

specificity of assays using original specimens as source of RNA was 100% (no cross-hybridization) with an overall sensitivity of 90.4% (151 correct positives and negatives out of a total of 167 samples).

Although no H5 influenza virus was observed in our sample collection (Tables 3 and 4), the usefulness of the H5 subtype specific assay is demonstrated by alignment analysis of known H5 influenza A virus with H5 primer/probe sequences and H5 assay sensitivity by template serial dilution. Shown in Fig. 1 (frame A) is alignment analysis of 22 H5 influenza A hemagglutinin sequences including an isolate from the first human H5 outbreak (1997) and subsequent outbreaks through 2006 with the H5 subtype specific, primer/probe sequences. Complete (100%) primer/probe, H5 virus sequence homology (avian and mammalian sources) was observed. Shown in Fig. 1 (frame B) is a representative profile of serially diluted H5 cRNA template (obtained from a human fatality) over an 8-log dilution range ( $10^{-9}$ – $10^{-16}$  g) corresponding to approximately 100 H5 cRNA target molecules ( $10^{-16}$  g). Serially diluted cRNA targets (type A, subtypes H1 and H3 and type B) exhibited very similar profiles to that shown in Fig. 1, frame B (data not shown).

## Conclusions

Influenza type specific assays described in this report detected all 16 known type A viruses including the recently discovered H16 strain<sup>27</sup> as well as both Yamagata and Victoria type B viruses. Using both the laboratory-based Lightcycler 2.0 instrument and its lightweight (50 lbs) version, the Ruggedized Advanced Pathogen Identification Device (RAPID), 347 archived primary clinical samples (throat swab/nasal wash, 180 cultured and 167 uncultured), were typed, i.e., influenza A or B and if A, which subtype. Of the 347 total samples evaluated, 278 were correctly identified as being influenza type A (222) or B (56), and all type A were subsequently subtyped as either H1, H3, or H5. Influenza negative (69) samples (Tables 3 and 4, 2 from the District of Columbia, 66 from Nepal and 1 from Texas, respectively) were subsequently confirmed as Coxsackie B, Adenovirus, Parainfluenzas 1, 2, and 3 or virus negative (data not shown). Sixteen of the 100 uncultured primary clinical specimens (Table 4) required subsequent culturing. Although rRT-PCR is capable of detecting the presence of nucleic acid from non-viable virus, this was not the case since all 16 primary samples were successfully cultured and identified suggesting target RNA degradation from prolonged storage at  $-80^{\circ}\text{C}$ , poor sample collection or lower sensitivity in clinical samples than that observed for serially diluted cRNA.

Assay specificity is underscored by the absence of any cross-reactivity, i.e., false positives (of particular note H5).



humans; however, the H1N2 strain is genetically similar to H1 New Caledonia-like viruses.<sup>29</sup> While H1N1 viruses were negligible during the 2004/05 season, an increasing number of human H1N1 viruses (A/New Caledonia-like strains) were detected worldwide during the 2005/06 season. Subsequently, during the 2006/07 season, an H1N1 drift variant constituting a new lineage represented by the A/Solomon Islands/3/2006 strain has emerged prompting a change in the 2007/08 H1 vaccine strain component. In similar fashion, the H3N2 virus co-circulating with H1N1 since 1977 has undergone 4 vaccine strain iterations since 1999.<sup>30</sup> The H1 and H3 subtype specific assays reported here were designed and empirically evaluated using 100 plus H1 and H3 vaccine and field strain sequences collected since 2001. Although H1 and H3 lineages exhibit genetic diversity, these changes do not occur within the H1 and H3 specific assay primer/probe binding regions and thus detection of genetically diverse viruses is not compromised.

Since 1997, genetic diversity has also been observed in H5 viruses in humans.<sup>31–34</sup> Human H5 outbreaks during 2004 and 2005 revealed two distinct clades of which the majority of human isolates characterized belonged to Clade 1. However, the number of reported H5 N1 Clade 2 human infections increased in late 2005. Although current H5 viruses have drifted from primer/probe sequences originally described by Spackman,<sup>23</sup> critical sequence analysis of human and avian sequences (22, cf. Fig. 1, panel A) used in this study revealed 100% primer/probe sequence homology supporting detection of both H5 Clades 1 and 2 (subclades 1, 2, and 3).<sup>31–34</sup> Furthermore, of particular note is the absence of H5 false positives observed in 167 original specimens and 180 cultures. However, due to empirical evaluation using a single H5 sample in this study, we cannot rule out the possibility of encountering false negatives using the H5 subtype specific assay. Thus, additional testing is needed to establish firmly the usefulness of the H5 specific assay described in this report.

Although the current FDA-approved LRN assays for influenza virus type A and H5 subtyping have been well-received, they do not (i) detect all 16 type A influenza viruses as well as both Yamagata and Victoria type B influenza lineages and (ii) differentiate currently circulating H1 and H3 influenza A viruses. Furthermore, the restricted nature and use of the LRN assay reagents in only a small number of LRN approved laboratories as well as restricted access to commercially available assays underscores the importance of needed additional assays for influenza virus detection in the event of a worldwide influenza outbreak.

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