CELLEX, INC.

QFlu® Combo Test

Key Symbols Use

	Catalogue Number
	Batch Code
	Expiration Date
	Temperature Limit
	Latex Free
	Caution
	Consult Instructions For Use

	In Vitro Diagnostic Medical Device
	Positive Control
	Contains Sufficient For <n> Tests
	Keep Away from Sunlight

QFlu™ Combo Test

For Diagnosis of Infection of Influenza Virus A & B and Detection of Drug Resistance

Cellex, Inc.

I. INTENDED USE

The QFlu® Combo Test is intended for use as an aid in diagnosis of infection with influenza Types A or B virus and determination of resistance to oseltamivir (Tamiflu[a]).

II. SUMMARY

Influenza illness is classically characterized by sudden onset of fever, chills, headache, myalgias, and non-productive cough. Epidemics of influenza typically occur during winter months with an estimated 114,000 hospitalizations[1] and about 36,000 deaths[2] per year in the U.S. Globally, influenza epidemics lead to 3-5 million cases of severe illness and 300,000-500,000 deaths annually[3]. Periodically, a new strain or variant of human influenza virus appears, leading to an influenza pandemic and dramatically increased numbers of severe illnesses and deaths from influenza-related complications.

Patients who are suspected of having influenza may benefit from treatment with an antiviral agent. Available is a new generation of therapeutic drugs and drug candidates targeting influenza viral neuraminidase[4-6], a critical enzyme for the life cycle of influenza Types A and B viruses[7]. Two neuraminidase inhibitors – Zanamivir and particularly oseltamivir – are the current mainstay of pharmacological intervention during an influenza epidemic or pandemic[4]; other neuraminidase inhibitor drug candidates such as Laninamivir and Peramivir can potentially play a similar role[8-10].

Emergence of drug resistant variants heightens the concern about resistance to these drugs[11-24]. Surveillance study showed that 8.6% of the circulating H1N1 influenza viruses in flu seasons between 2004 and 2008 were resistant to oseltamivir[25]. Widespread oseltamivir-resistance was found among the seasonal influenza virus A/H1N1 during the 2008/2009 flu season in the U.S.[26-29]. A rapid diagnostic tool for detection of resistance to influenza antivirals could be useful in managing the use of these influenza antivirals.

The QFlu Combo Test (QFlu Test) is an *in vitro* diagnostic test designed for use in diagnosis of influenza and, if positive, determination of resistance to antivirals targeting influenza viral neuraminidase.

III. PRINCIPLE OF DETECTION

The QFlu Test detects influenza viral neuraminidase activity. A neuraminidase-based assay is unique in that it detects the enzymatic activity of a viral component, rather than an epitopic sequence in an immunoassay or nucleic acid sequences in a molecular assay and, consequently, is less susceptible to genetic changes of the virus. As influenza viral neuraminidase is also a drug target, a neuraminidase assay can be used for detection of resistance to an antiviral targeting the enzyme. The QFlu Combo Test is designed to simultaneously detect the presence of influenza virus Types A or B in a sample and their status of susceptibility to oseltamivir.

The QFlu Test uses a biochemiluminescence substrate that favors influenza viral neuraminidase, which can hydrolyze substrates that contain alpha-ketosidically linked N-acetylneuraminic acid (Neu5Ac). Other viruses such as parainfluenza and mumps viruses also possess surface enzymes with neuraminidase activity. However, unlike influenza viral neuraminidase, which primarily cleaves the alpha – 2,6 bond, neuraminidase enzymes from these Paramyxovirus viruses favors alpha – 2,3 linkage[30-32]. Thus, paramyxovirus may not be detected in the QFlu Test. Although certain bacterial species, such as *clostridium perfringens*, possess neuraminidase that has alpha – 2, 6 activity[33-38], the substrate used in the QFlu Test exhibits specificity towards influenza virus Types A and B neuraminidases, therefore minimizing potential interference from these bacterial species.

The QFlu Combo Test contains two key reagents, Reagents I and II, which are identical except that Reagent II contains an antiviral targeting the influenza viral neuraminidase. The QFlu Test is simple and rapid. The assay involves 1) sample collection, 2) elution of the sample in a sample elution buffer, 3) addition of the sample to Reagents I and II Vials, 4) incubation at room temperature for 15 minutes, and 5) detection of the signal with an analyzer (60 seconds). The entire process can be completed within 20 minutes.

Presence of influenza virus, hence viral neuraminidase, enables the generation of light signal that is detected with the analyzer. The signal from Reagent I is used to diagnose influenza whereas the signal ratio of Reagent II to Reagent I is used to determine drug resistance or susceptibility.

An R-Factor, which is 10 times the ratio of Reagent II signal over Reagent I signal, is used to indicate drug resistance of a virus. Cutoff values for Reagent I signal and R-Factor are set for influenza diagnosis and oseltamivir resistance, respectively, based on data from clinical and analytical studies.

IV. REAGENTS AND SUPPLIES

A. KIT COMPONENTS

The QFlu Combo Test kit contains the following components:

	Catalog No.	20K500
	Number of Tests/Kit	**20**
Kit Component	QFlu Reagent I Pouch (20 Tests/Pouch)	1
	QFlu Reagent II Pouch (20 Tests/Pouch)	1
	1X Q-Sample Buffer Vial	20
	Exact Pipette (0.25 mL)	20
	Positive Control 1 (PC-1)	1
	Positive Control 2 (PC-2)	1

Note: Use 1X Q-Sample Buffer as the negative control.

B. STORAGE CONDITIONS

The Reagent Pouch and control pouch or the entire kit should be stored at 2-8°C.

C. SUPPLIES NOT PROVIDED (PURCHASED SEPARATELY)

- Helios 200 Analyzer
- 2X Q-Sample Buffer
- Nasopharyngeal Sample Collection Swabs (may be purchased from other suppliers)
- Heat Block (optional)

V. SAMPLE COLLECTION, TRANSPORT AND STORAGE

A. SAMPLE COLLECTION

Sample collection from patients is critical for accurate diagnoses. Collect nasopharyngeal (NG) using a NG swab (not provided as part of the test kit) as follows:

1. Remove any mucus that is blocking the nasal passage.

[a] Tamiflu is a trademark owned by Roche.

2. Estimate the distance from the base of the nose to the front of the ear and insert the swab only ½ this distance.
3. Tilt the patient's head back.
4. Gently insert the swabs along the medial part of the septum (coughing may occur), rotate the swab and remove it;
5. Insert the swab into 1X Q-Sample Buffer Vial and tightly close the cap; label with patient ID.
6. The sample with the swab can be kept at 2-8°C for up to 96 hours

B. SAMPLE TRANSPORT

Specimens in 1X Q-Sample Buffer may be transported on ice. Refer to supplier's instruction for samples collected in other media.

C. SAMPLE STORAGE

Specimens in 1X Q-Sample Buffer can be stored at 2-8°C for up to 96 hours. Follow supplier's instruction for samples collected in other media.

VI. DETECTION PROTOCOL

A. DETECTION PROTOCOL FOR SAMPLES COLLECTED IN 1X Q-SAMPLE BUFFER

Step 1 – Add 250 µL of the sample in 1X Q-Sample Buffer to **Reagents I and II** Vials.

Step 2 – Incubate at room temperate for 15 minutes.

Step 3 – Place the reagent vials in Helios 200 Analyzer and close the chamber cap to initiate signal measurement. Refer to Section VIII for instruction to operate the Helios 200.

Step 4 – Record sample information and test results for permanent record. See next section for result interpretation guide.

B. PREPARATION OF SAMPLES COLLECTED IN OTHER SAMPLE MEDIA (VTM, Hank's Salts, and M4)

1. Mix a sample with an equal volume (e.g., 0.5 mL) of 2X Q-Sample Buffer. Save the remaining sample in the medium for culture use if necessary.
2. Follow Steps 1-4 of the detection protocol.

Note: Q-Sample Buffer contains a detergent, which inactivates the virus. Consequently, the virus in Q-Sample Buffer can no longer be recovered for culture.

C. TESTING THE POSITIVE CONTROLS

1. Add the PC-1 or PC-2 reagent bead into a 1X Q-Sample Buffer Vial and mix to dissolve the reagent.
2. Follow Steps 1-4 of the detection protocol.
3. Expected results are provided in **Table 1**.

Table 1 | Expected Positive Control Test Results

	QFlu Reagent I Reading on H200	R-Factor
PC-1	> 1000	< 2.40
PC-2	> 1000	≥ 2.40

VII. RESULT INTERPRETATION

The Helios 200 Analyzer measures the signal and performs interpretation of test results. There are four possible test results (**Table 2**), which are based on two cutoff values: a) the influenza diagnosis cutoff (Reagent I Reading ≥ 220 for influenza) and b) Oseltamivir (OC) susceptibility cutoff (R-Factor ≥ 2.40 for drug resistance).

When Reagent I signal falls in the equivocal zone (≥ 220, but <917), the R-Factor cannot be reliably used to indicate drug susceptibility or resistance, resulting an equivocal result for drug resistance determination.

$$\text{R-Factor} = 10 \times \frac{\text{Reagent II Signal}}{\text{Reagent I Signal}}$$

Table 2 Test Result Interpretation Guide

	Reagent I Reading	R-Factor	Interpretation
1	<220	N/A	Flu Negative
2	> 916	≥ 2.40	Flu Positive, OC Resistant
3	> 916	< 2.40	Flu Positive, OC Susceptible
4	220 - 916	Any	Flu Positive, Equivocal Resistance Determination

VIII. OPERATION OF HELIOS 200 ANALYZER

An abbreviated operation instruction for Helios 200 is provided in this section. Refer to Operation Manual of Helios 200 for detailed and up-to-date instruction.

1. Calibrate the Analyzer

   When the Analyzer is first turned on, it automatically undergoes self-calibration.

2. Select Run Tests and then QFlu Combo Test

3. Input Sample ID when necessary

4. Perform a Measurement

   Follow the instruction on the screen:

   - Open the cap;
   - Insert Reagent I Vial to the Left Chamber;
   - Insert Reagent II Vial to the Right Chamber;
   - Close the cap firmly;
   - Touch the "Start" to begin measurement;

5. Record the Test Results

   - Record the test results;
   - Helios 200 Analyzer can temporarily store up to 200 sample testing data.

IX. PERFORMANCE CHARACTERISTICS

A. REACTION KINETICS

Three levels of two influenza virus strains, an Oseltamivir susceptible (wild type) and a resistant strain, were tested (**Table 3**). Both strains of virus were 2009 H1N1 Pandemic strains. The Oseltamivir resistant variant, A/H1N1/NC/39/2009, carries a H275Y mutation, which confers Oseltamivir resistant phenotype. In the presence of influenza virus, signal intensity from Reagent I of the QFlu Test increased rapidly in the first 5 to 10 minutes, followed by a plateau period that lasted at least 120 minutes (**Fig 1**).

Table 3 | Sample Panel Used in Kinetics Study

Panel Member	Virus Strain	OC Resistance	Virus Level	Mean	%CV
1	A/CA/07/2009	S	H	11,666	2.46
2	A/NC/39/2009	R	H	11,565	2.31
3	A/CA/07/2009	S	M	3,860	1.71
4	A/NC/39/2009	R	M	2,960	2.45
5	A/CA/07/2009	S	L	561	6.65
6	A/NC/39/2009	R	L	416	6.24
7	Negative	N/A	N/A	N/A	N/A

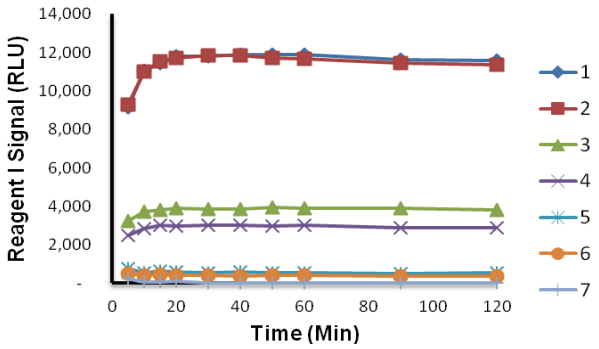

Fig 1 | Reaction Kinetics
Reactions were initiated by adding influenza virus to the detection mix. The reaction kinetics over a period of 120 minutes was recorded using a Helios 200.

Samples with neuraminidase activities above the equivocal zone (Panel Members 1-4, **Table 3**), both A/CA/07/2009 (Wild Type-WT) and A/NC/37/2009 (275Y Mutant) virus strains, were tested with both Reagent I and II of the QFlu Test. The R-Factor values were calculated for various time points up to 120 min. The R-Factor stabilized after 15 minutes. The R-Factor values for the wild type (WT) virus were well separated from those for the OC-resistant mutant for both virus concentrations throughout the reaction period of up to 120 min (**Fig 2**).

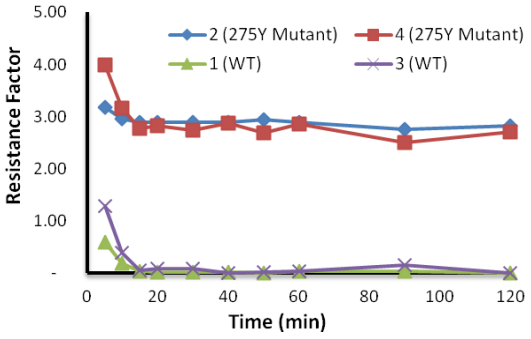

Fig 2 | Inhibition Kinetics
Reactions were initiated by adding influenza virus to QFlu Reagents I and II. R-Factor values were computed using signals collected over a period of 120 minutes.

B. PRECISION

1. Site-to-Site Reproducibility

A study panel comprising samples with oseltamivir resistant or susceptible virus at low to medium levels of titers were tested along with a negative sample in three sites over a period of five days. Two operators were involved in the study in each site. Each operator tested three replicates each day.

Presence or absence of influenza virus was correctly determined for all samples and replicates (**Table 4**). OC resistance status was correctly determined for at least 98.89% of the replicates (95% CI: 94.03% - 99.73%) for a sample with mean Reagent I readings above the equivocal zone (**Table 4**). The coefficients of variation of Reagent I RLU for all influenza virus positive samples were less than 30% (**Table 4**). As expected, higher variability was observed for those samples with lower RLU such as the negative sample or Reagent II RLU for susceptible virus samples.

Table 4 | Site-to-Site Reproducibility

		OC Resistant Virus			OC Susceptible Virus		NC
Sample		1*	2	3	4*	5	6
	N	90	90	90	89	90	90
Reagent I RLU	Mean	371	1276	5402	687	2976	58
	SD	78	197	1006	199	642	45
	%CV	20.99	15.26	18.63	29.02	21.56	78.88
R-Factor	Mean	5.46	4.08	3.57	1.66	0.42	N/A
	SD	1.91	0.95	0.65	1.2	0.28	N/A
	%CV	34.98	23.24	18.23	72.67	66.29	N/A
% Flu Positive		100	100	100	100	100	0
% OC Resistant		94.44	98.89	100.00	21.35	0	N/A

*Positive samples with signal within the equivocal zone for drug resistance determination.

2. Within Site Repeatability

The same study panel used for the Site-to-Site Reproducibility Study was used for this study. The samples were tested over a period of 12 days. Two runs and two replicates per run were performed daily.

Presence or absence of influenza virus was correctly identified for 100% of the replicates for all samples (95% CI: 92.75% - 99.95%) (**Table 5**). Except for the negative sample and Sample #4, for which the Reagent I signal was within the equivocal zone, drug resistance status was correctly determined for 100% of the replicates for all samples (**Table 5**).

Table 5 | Within Site Repeatability

		OC Resistant Virus			OC Susceptible Virus		NC
Sample		1*	2	3	4*	5	6
	N	48	48	48	48	48	48
Reagent I RLU	Mean	373	1009	3933	724	3357	101
	SD	82	198	1003	228	352	34
	%CV	21.91	19.68	25.50	31.49	10.48	33.59
R-Factor	Mean	6.02	4.55	3.55	2.85	0.70	N/A
	SD	1.65	1.15	0.43	1.56	0.38	N/A
	%CV	27.40	25.37	12.22	54.87	54.82	N/A
% Flu Positive		100	100	100	100	100	0
% OC Resistant		100	100	100	52.08	0	N/A

*Positive samples with signal within the equivocal zone for drug resistance determination.

C. LINEARITY

Samples containing 2.83 to 5.06 log $TCID_{50}$ units/mL of influenza virus (A/CA/07/2009; wild type) were tested. The correlation

coefficient (R^2) over this virus concentration range (2.23 log units) was 0.9967 (95% CI: 0.9690-1.0; **Fig 3**). Similar linear range and linearity were observed for the oseltamivir resistant virus A/NC/39/2009.

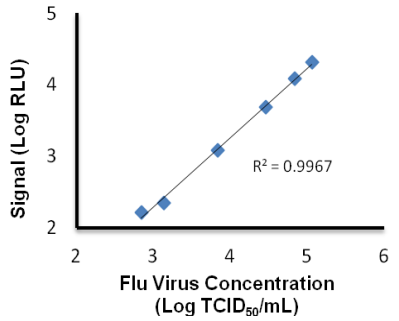

Fig 3 | Linearity
A scattering plot between signal (relative light units, RLU) and influenza virus concentrations.

D. LIMITS OF DETECTION (LOD)

Samples of two influenza virus strains (A/CA/07/2009 and A/NC/37/2009) approaching the limit of detection were tested in 20 replicates. LOD is defined as the lowest concentration tested, which gave a positivity rate of at least 95%. The LOD for influenza virus detection was 995 and 953 $TCID_{50}$/mL for A/CA/07/2009 and A/NC/39/2009, respectively (**Table 6**). The LOD for oseltamivir resistance detection was 1,326 and 953 $TCID_{50}$/mL for A/CA/07/2009 and A/NC/39/2009, respectively (**Table 6**).

Table 6 | Limits of Detection

Panel Member		**1**	**2**	**3**	**4**	**5**
Virus Strain		A/CA/07/2009			A/NC/39/2009	
OC Resistance Status		Susceptible			Resistant	
Virus Concentration ($TCID_{50}$/mL)		663	995	1,326	953	1,271
S/CO	Mean	0.98	1.58	1.96	1.15	1.87
	%CV	13.81	7.98	6.59	8.36	3.45
R Factor	Mean	3.0	1.5	0.8	5.5	4.4
	%CV	26.98	35.09	61.92	15.5	9.06
Percentile	Flu Positive	45%	**100%**	100%	**100%**	100%
	OC Resistant	90%	10%	**0%**	**100%**	100%

E. COMPARATIVE ANALYTICAL SENSITIVITY

To compare the analytical sensitivity of the QFlu Test with an FDA-approved rapid antigen test, a Type A and a Type B virus strains were sequentially diluted and tested with both tests. This study showed that the QFlu Test is approximately 100 and 5 times as sensitive as the FDA approved rapid influenza antigen test for A/CA/07/2009 and B/NC/82/2009, respectively (**Table 7**).

Table 7 | Comparative Analytical Sensitivity

		Dilution				
		$1:10^2$	$1:10^3$	$1:10^{3.7}$	$1:10^4$	$1:10^5$
A/CA/07/09	RLU	25,973	2,786	459	238	0
	S/CO	117.00	12.55	2.07	1.07	0.00
	Pos/Neg	Pos	Pos	Pos	Pos	Neg
	Comparison Test	Pos	Neg	Neg	Neg	NT
B/NC/82/09	RLU	13,044	1,409	267	151	0
	S/CO	58.76	6.35	1.20	0.68	0.00
	Pos/Neg	Pos	Pos	Pos	Neg	Neg
	Comparison Test	Pos	Pos	Neg	Neg	NT

F. ANALYTICAL REACTIVITY (INCLUSIVITY)

Various strains of influenza virus collected from previous years were tested in triplicates at concentrations approaching the limit of detection (**Table 8**). All signals were above the cutoff. As the signals were within the equivocal zone for drug susceptibility detection, the R-Factor values for some of the samples were above the drug resistance cutoff. These samples were retested at a higher concentration, which resulted in R-Factor values below resistance cutoff indicative of drug susceptibility of these virus strains (**Table 8**). The data is consistent with the expected susceptibility to oseltamivir of these viruses.

Table 8 | Analytical Reactivity

Virus Strain	$TCID_{50}$/mL or $CEID_{50}$/mL	Reagent I (RLU)	Mean S/CO	Mean R-Factor
A/PR/8/34	0.8	284.00	1.29	1.77
A/FM/1/47	0.07	283.33	1.29	1.79
A/NWS/33	5,330	244.67	1.11	1.80
A/Denver/1/57	53,300	270.67	1.23	1.00
A/New Jersey/8/76	741	284.67	1.29	2.41
	1482	590.33	2.66	1.50
A/Port Chalmers/1/73	6,846	265.00	1.20	2.33
	12,702	459.67	2.09	1.63
A/Hong Kong/8/68	2,330	384.00	1.75	0.66
A/Aichi/2/68	13	282.33	1.28	0.48
A/Victoria/3/75	158	290.33	1.31	1.81
B/Lee/40	2.50	287.67	1.30	2.34
	166.67	777.67	3.53	1.68
B/Allen/45	1.98	280.67	1.28	1.83
B/GL/1739/54	0.11	285.00	1.30	3.50
	0.27	659.33	3.00	0.92
B/Taiwan/2/62	8.90	259.67	1.18	2.43
	29.67	795.33	3.62	0.85
B/Hong Kong/5/72	528	312.67	1.42	2.08
B/Maryland/1/59	1.48	305.00	1.39	3.16
	2.97	689.33	3.13	0.87

G. ASSAY SPECIFICITY AND CROSS-REACTIVITY

1. Potentially Interfering Substances

The following substances were tested and found no interference with the QFlu test: Whole blood with EDTA as anti-coagulant (0.25%), Mucin (0.25%), Phenylephrine (0.1%), Oxymetazoline

(0.005%), Sodium chloride with preservative (10%), Dexamethasone (0.5 mg/mL), Flunisolide (0.5 mg/mL), Beclomethasone (0.5 mg/mL), Triamcinolone (0.5 mg/mL), Fluticasone (0.5 mg/mL), Menthol (0.5 mg/mL), Tobramycin (0.5 mg/mL), Nasal Gel (10%), and Benzocaine (0.05 mg/mL).

2. Other Viruses

The following viruses were tested and found no interference with the QFlu test: Human Adenovirus Type 1 ($5x10^{5.5}$ $TCID_{50}$/mL), Human Adenovirus Type 7 ($5x10^{4.75}$ $TCID_{50}$/mL), Human Coronavirus ($1.6x10^{5}$ $TCID_{50}$/mL), Human Herpesvirus Type 4 ($5x10^{3.5}$ $TCID_{50}$/mL), Human Herpesvirus Type 5 ($5x10^{3.5}$ $TCID_{50}$/mL), Human Enterovirus ($1.6x10^{7}$ $TCID_{50}$/mL), Human Parainfluenza Type 2 ($1x10^{7}$ $TCID_{50}$/mL), Measles ($3.4x10^{3}$ $TCID_{50}$/mL), Human Syncytial Virus (RSV) and Rhinovirus ($5x10^{5.5}$ $TCID_{50}$/mL).

3. Microbes

The following microbial species were tested and found no interference with the QFlu Combo Test: *Chlamydia pneumoniae* ($5x10^{4.5}$ $TCID_{50}$/mL), *E. coli* ($1x10^{6}$ CFU/mL), *Mycoplasma pneumoniae* ($2x10^{5}$ CFU/mL), *Streptococcus aureus* ($1x10^{6}$ CFU/mL), *Streptococcus epidermidis* ($1x10^{6}$ CFU/mL), *Streptococcus pyogenes* ($1.7x10^{6}$ CFU/mL), *Haemophilus influenza* ($1.2x10^{6}$ CFU/mL), *Neisseria spp*. ($1.5x10^{6}$ CFU/mL), *Streptococcus salivarius* ($2.5x10^{6}$ CFU/mL), *Neisseria meningitidis* ($3.7x10^{6}$ CFU/mL), Moraxella catarrhalis ($3.8x10^{6}$ CFU/mL), and *Streptococcus pneumoniae* ($1.4x10^{4}$ CFU/mL). *Corynebacterium sp* (1.3 $x10^{6}$ CFU/mL), *Lactobacillus sp* ($1.8x10^{6}$ CFU/mL), *Pseudomonas aeruginosa* ($5.6x10^{6}$ CFU/mL). When tested at higher concentration, *e.g*., 10^{6} CFU/mL, *Streptococcus pneumoniae* resulted in noticeable increase in non-specific signal.

H. SAMPLE MATRICES AND STORAGE CONDITIONS

1. Sample Matrices

Samples collected in virus transport media were tested after dilution into 5X Q-Sample Buffer. Compared to samples directly eluted in 1X Q-Sample Buffer, which requires no dilution, at least 89% of the neuraminidase activity was detected for samples in UTM or M4 medium. However, only 59% activity was detected in samples collected in virus transport medium (VTM) containing DMEM/F12 (1:1) and 1% BSA.

2. Storage Conditions

More than 80% and 71% neuraminidase activity was retained after storage for up to 96 hours (4 days) at 2-8°C or room temperature, respectively, in 1X Q-Sample Buffer, VTM with BSA, UTM or M4 medium (**Table 9**).

Table 9 | Stability of Samples in Various Sample Matrices

Time (Hr)	Temp	Q-Sample Buffer	VTM with BSA	UTM	M4
0	N/A	100	100	100	100
24	2-8°C	88.70	91.83	83.66	102.32
	RT	90.12	78.19	76.11	83.82
48	2-8°C	88.07	87.07	88.97	99.12
	RT	87.03	77.08	82.75	88.57
72	2-8°C	84.30	82.87	82.30	100.45
	RT	87.69	78.91	78.10	82.87
96	2-8°C	80.64	87.41	87.66	95.97
	RT	87.95	75.86	71.59	82.89

3. Freeze/Thaw Cycles

Samples were stored at -70°C and tested after each freeze/thaw cycle. The retained activity of a sample is expressed as a percentage of that before freezing. Freezing of samples in 1X Q-Sample Buffer caused significant loss of neuraminidase activity (**Table 10**). At least 77.06% activity could be retained after three freeze/thaw cycles when stored in VGM (Virus Growth Medium), UTM or M4 (**Table 10**).

Table 10 | Stability of Samples after Freeze/Thaw

Matrices	Virus	Number of Freeze/Thaw Cycles			
		0	1	2	3
Q-Sample Buffer	A/CA/07/09	100	29.67	31.58	31.34
	A/NC/39/09	100	58.96	64.68	64.43
VGM	A/CA/07/09	100	85.43	89.80	82.15
	A/NC/39/09	100	77.06	82.03	81.64
UTM	A/CA/07/09	100	90.17	94.71	100.19
	A/NC/39/09	100	88.96	88.96	93.60
M4	A/CA/07/09	100	91.67	90.28	102.08
	A/NC/39/09	100	96.24	90.23	95.49

I. DETECTION OF SAMPLES WITH MIXED VIRUSES

An oseltamivir resistant virus (A/NC/39/2009; H275Y mutant) was mixed with an increasing amount (in terms of neuraminidase activity) of an oseltamivir susceptible virus (A/CA/07/2009; wild type - WT) and tested in triplicate with the QFlu test. The value of R-Factor decreased to below the resistance cutoff of 2.40 when the proportion of susceptible virus in the sample increased to more than 19.64% (**Table 11**).

Table 11 | Detection of Samples with Mixed Viruses

Sample		R-Factor			
		Replicate			
Number	% WT Virus	1	2	3	**Mean**
1	0	3.1	3.0	2.8	3.0
2	10	2.7	2.5	2.6	2.6
3	20	2.5	2.2	2.3	2.3
4	30	2.1	2.2	2.3	2.2
5	40	1.6	1.8	1.9	1.8
6	50	1.6	1.5	1.6	1.6
7	60	1.3	1.5	1.4	1.4
8	70	1.0	1.0	1.0	1.0
9	80	0.7	0.7	0.6	0.7
10	90	0.5	0.4	0.4	0.5
11	100	0.2	0.2	0.1	0.2

J. CLINICAL PERFORMANCE FOR DRUG RESISTANCE DETECTION

1. IC_{50} for Drug Resistance Determination

IC_{50} is an antiviral concentration at which 50% of the neuraminidase activity is inhibited. It is commonly used to indicate drug susceptibility of an influenza virus. The IC_{50} cutoff value for OC was determined by using a group of virus strains consisting of 08/09 seasonal A/H1N1, 08/09 seasonal A/H3N2, 07/08 A/H1N1 and Type B virus from 2009 or 2011. Most of the 2008/2009 seasonal H1N1 isolates are known to carry the H274Y mutation, which confers resistance to oseltamivir[25-28]. Oseltamivir IC_{50} values were determined using the QFlu Neuraminidase Inhibition (NI) Assay.

As Type B virus is known to be less susceptible to oseltamivir, the IC_{50} values from the Type B virus were used to set the oseltamivir

resistance cutoff value, which is defined as the IC_{50} value at 3 standard deviations (SD) above the mean IC_{50} value for Type B virus, *i.e.*, 38.04 nM. A virus with an IC_{50} value 3 standard deviations above the mean is considered an "outlier", *i.e.*, a drug resistant variant[25].

Based on this IC_{50} cutoff, all 08/09 H1N1 isolates were resistant while all 08/09 H3N2, 07/08 H1N1 and Type B virus were susceptible to oseltamivir (**Table 12**), as expected. The sensitivity and specificity are 100% (95% CI: 96.45% - 99.98%) and 100% (95% CI: 96.34% - 99.97%), respectively.

Table 12 | Oseltamivir IC_{50} Profiles

Oseltamivir Susceptibility		Resistant	Susceptible		
Strain		08/09 H1N1	07/08 H1N1	08/09 H3N2	Type B
IC_{50} (nM)	N	104	9	35	54
	Mean	244.71	2.64	1.55	18.69
	SD	133.25	1.86	1.10	6.45
	Range	79.58-717.97	1.80-7.26	0.40-7.10	5.46-36.24
% Susceptible		0	100	100	100

For comparison, Zanamivir IC_{50} values were also similarly determined for these isolates using the QFlu NI Assay (**Table 13**). Using an IC_{50} cutoff value set at three (3) standard deviations above the mean for Type B viruses, *i.e.*, 17.13 nM Zanamivir, all but one isolates had IC_{50} value below the cutoff. Assuming all virus were susceptible to Zanamivir, the specificity was 99.49% (95% CI: 97.28%-99.88%) for the IC_{50} based assay.

Table 13 | Zanamivir IC_{50} Profiles

Virus Strain		08/09 H1N1	07/08 H1N1	08/09 H3N2	Type B
IC_{50} (nM)	N	104	9	35	54
	Mean	1.17	3.51	1.87	7.14
	SD	0.30	4.23	2.08	3.33
	Range	0.50-13.17	1.35-7.26	0.84-13.47	2.86-22.57

2. QFlu Combo Test Performance with Clinical Isolates

Oseltamivir susceptibility of the clinical isolates described above was assessed using the QFlu test. The R-Factor values for oseltamivir were calculated for each sample. Based on an R-Factor cutoff value of 2.40 (**Table 2**), all 08/09 H1N1 isolates were resistant to oseltamivir whereas all 07/08 H1N1, 08/09 H3N2 and Type B isolates were susceptible to Oseltamivir (**Table 14**).

Table 14 | Detection of Oseltamivir Susceptibility with QFlu Test

Oseltamivir Resistance Status by IC_{50}			Resistant Virus	Susceptible Virus		
Strain			08/09 H1N1	07/08 H1N1	08/09 H3N2	Type B
QFlu Test	R-Factor (Oseltamivir)	N	104	9	35	55
		Mean	4.29	0.23	0.31	0.74
		SD	0.39	0.11	0.30	0.24
		Range	3.75-6.02	0.05-0.38	0.83-1.29	0.42-1.67
		% Susceptible	0	100	100	100
	Sensitivity		100% (95% CI: 96.55% - 99.98%)			
	Specificity		100% (95% CI: 96.34% - 99.98%),			

By comparison with the IC_{50}-based drug resistance determination, the sensitivity and specificity of the QFlu test for Oseltamivir resistance detection were 100% (95% CI: 96.55% - 99.98%) and 100% (95% CI: 96.34% - 99.98%), respectively (**Table 14**).

The R-Factor values of the QFlu Test were plotted against the oseltamivir IC_{50} values for these isolates (**Fig 4**). The IC_{50} and R-Factor values were correlated well and could be readily separated into two groups, *i.e.,* a group with high IC_{50} and high R-Factor values indicative of oseltamivir resistance and a group with low IC_{50} and R-Factor values indicative of oseltamivir susceptibility. Thus, like the neuraminidase inhibition assay, which measures the IC_{50}, the QFlu Combo Test can be used to measure drug resistance.

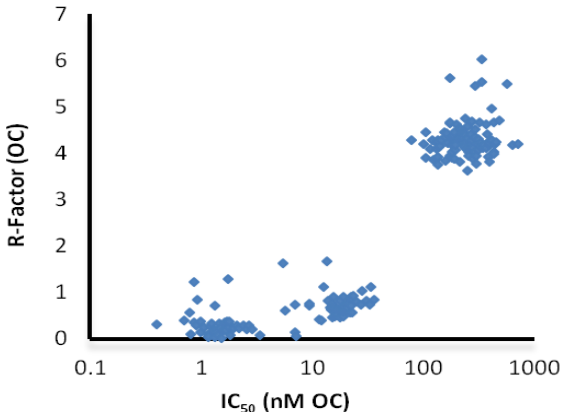

Fig 4| Correlation between Oseltamivir IC_{50} and R-Factor Values. A scattering plot between Oseltamivir (OC) and R-Factor values and IC_{50} values (nM).

For comparison, a QFlu test that was similarly formulated for detection of Zanamivir resistance was used to determine Zanamivir susceptibility of these virus isolates. Based on an R-Factor cutoff value of 2.40, all virus isolates were susceptible to Zanamivir, giving a specificity of 100% (95% CI: 98.12% - 99.99%) (**Table 15**); however, the sensitivity cannot be estimated as there was no Zanamivir-resistant isolates in the study sample population.

Table 15 | Detection of Zanamivir Susceptibility with QFlu Test

Zanamivir Resistance Status by IC_{50}			Presumed Susceptible Virus			
Strain			08/09 H1N1	07/08 H1N1	08/09 H3N2	Type B
QFlu	R-Factor (Zanamivir)	N	104	9	35	54
		Mean	0.16	0.19	0.33	0.17
		SD	0.18	0.08	0.38	0.20
		Range	0-1.17	0.06-0.26	0-1.66	0-1.17
		Mean (Total)	0.19			
		SD (Total)	0.23			
		Range (Total)	0-1.66			

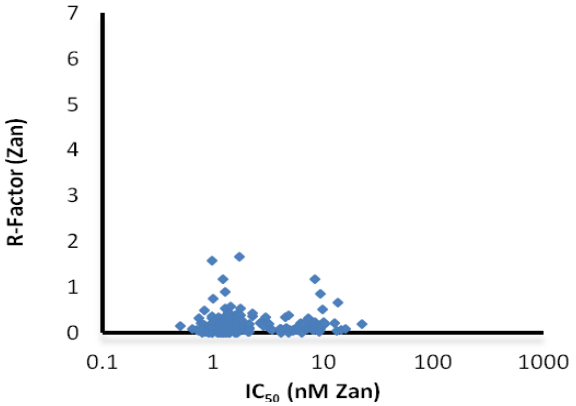

Fig 5| Correlation between IC_{50} and R-Factor Values
A scattering plot between Zanamivir (Zan) R-Factor values and IC_{50} values (nM) in Log units.

The R-Factor values of the QFlu Test for Zanamivir were plotted against the Zanamivir IC_{50} values for these isolates (**Fig 5**). Compared to the oseltamivir plot (**Fig 4**), all isolates were clustered into one group, *i.e.,* those with low and IC_{50} and R-Factor values, indicating that there was no virus isolate that was highly resistant to Zanamivir in this group of samples as estimated by either the IC_{50} assay or the QFlu test.

3. QFlu Test Performance with Clinical Specimens

A total of 460 patients who exhibited flu-like symptoms were enrolled in a study during the 2011/2012 influenza season in U.S. Two nasopharyngeal samples were collected from each patient, one from each nostril. One of the collected samples was used for culture whereas the other used for QFlu test for oseltamivir resistance detection. Influenza virus isolates were recovered from thirty-three (33) of the patient samples and determined for oseltamivir IC_{50} using the QFlu NI Assay.

The mean IC_{50} for the Type A virus isolates (N=23) was 2.67 nM oseltamivir, ranging from 0.98 nM to 8.43 nM whereas the mean IC_{50} for Type B virus isolates (N=10) was 20.79 nM, ranging from 9.69 nM to 34.98 nM (**Table 16**). All of these virus isolates have IC_{50} less than the cutoff IC_{50} of 38.04 nM established for oseltamivir. Thus all isolates were susceptible to oseltamivir as determined by the inhibition assay.

Of the thirty-three (33) culture positive samples, twenty-three (23) or 69.70% were positive for influenza by QFlu Test when tested with the clinical samples; however, signal intensity of the QFlu test for five of the 23 samples was in the equivocal zone (between 220 and 916) for oseltamivir drug resistance determination. Of the eighteen samples with unequivocal signal intensity, the mean R-Factor for Type A virus was 0.62 ranging from 0 to 2.37 whereas the mean R-Factor for Type B virus was 1.16 ranging from 0 to 2.4 (**Table 16**). Two of the samples, one Type A and another Type B, had R-Factor values slightly above the cutoff of 2.40 (2.34 and 2.37, respectively).

Thus, when unequivocal R-Factor was obtained, the specificity of the QFlu Test for oseltamivir resistance detection was 90% (95% CI: 69.62% - 96.95%) when compared to that of an IC_{50} assay. The sensitivity could not be estimated from this group of samples as all viruses were oseltamivir susceptible.

Table 16 | Detection of Oseltamivir Susceptibility with QFlu Test Using Clinical Specimens

Virus Type	Inhibition Assay (IC_{50}, nM Oseltamivir)		QFlu Test (R-Factor)	
Type A Virus (N=11)	Mean	2.16	Mean	0.68
	SD	0.75	SD	0.81
	Range	1.03-3.32	Range	0-2.34
Type B Virus (N=7)	Mean	22.66	Mean	1.26
	SD	8.42	SD	0.73
	Range	14.69-34.98	Range	0.25 -2.37
Total (N=18)	Mean	10.13	Mean	0.91
	SD	11.45	SD	0.81
	Range	1.03-34.98	Range	0 -2.37

Zanamivir IC_{50} was also determined for these isolates from the clinical study. Similar to oseltamivir IC_{50} for this group of isolates, Zanamivir IC_{50} values were low, ranging from 0.66 to 8.86 nM for Type A virus and from 1.26-6.94 nM for Type B virus (**Table 17**). The low Zanamivir IC_{50} values indicate that these viruses are susceptible to Zanamivir as well.

Table 17 | Zanamivir Susceptibility of the Clinical Isolates Measured with the Inhibition Assay

Virus Type	Inhibition Assay (IC_{50}, nM Zanamivir)	
Type A Virus	N	23
	Mean	1.56
	SD	1.63
	Range	0.66-8.86
Type B Virus	N	10
	Mean	4.43
	SD	1.55
	Range	1.26-6.94

K. CLINICAL PERFORMANCE FOR INFLUENZA DIAGNOSIS

Test results of QFlu Reagent I in the QFlu Combo Test alone can be used for diagnosis of influenza. When the signal of QFlu Reagent I is at or above 220, presence of influenza virus in the sample is indicated. Studies were carried out to assess the clinical utility of the QFlu Reagent I for diagnosis of influenza. The data from these studies are present below.

1. Clinical Sensitivity and Specificity for Influenza Diagnosis

A total of 1974 throat swabs were collected from patients exhibiting symptoms of upper respiratory infection. All samples were tested with RT-PCR (reverse transcription polymerase chain reactions). Of these samples, 678 were RT-PCR positive and 1,296 were RT-PCR negative.

These samples were tested using the QFlu Reagent I of the QFlu Combo Test (herein referred to as QFlu Test). Of the 678 RT-PCR-positive samples, 560 were tested positive with the QFlu test whereas 1,241 of the RT-PCR negative samples (N=1296) were tested negative with the QFlu test (**Table 18**). The sensitivity and specificity of the QFlu Test for influenza diagnosis are 82.60% (95% CI: 80.01-84.80%) and 95.76% (95% CI: 95.30-96.30%), respectively.

Table 18 | Clinical Sensitivity and Specificity for Influenza Diagnosis

		QFlu Test		**N**
		Positive	Negative	
RT-PCR	Positive	560	118	678
	Negative	55	1241	1296
N		615	1359	**1974**
Sensitivity: 82.60% (95% CI: 80.01-84.80%)				
Specificity: 95.76% (95% CI: 95.30-96.30%)				

The 615 PCR-positive samples consist of 91 Type B virus, 173 seasonal A/H1N1, 89 pandemic A/H1N1 (A/pH1N1), 305 seasonal A/H3N2 and 20 A/H7N9. Except for H7N9 virus, which had only 20 samples, all flu virus types/subtypes showed similar detection rate by the QFlu Test (**Table 19**).

Table 19 | Composition of Flu Positive Samples

Virus Type/Subtype	**RT-PCR**	**QFlu Test**	
	Positive (N)	**Positive (N)**	**% Detected**
Type B	91	74	81.32
A/H1N1	173	142	82.08
A/pH1N1	89	73	82.02
A/H3N2	305	257	84.26
A/H7N9	20	14	70.00
Subtotal (N)	**678**	**560**	**82.60**

2. Comparison with a Lateral Flow-Based Test

To compare the performance characteristics of QFlu Test with those of a lateral flow test, 97 throat swab samples were randomly selected and tested with RT-PCR, QFlu Test and a lateral flow based test. In comparison with RT-PCR, the QFlu Test detected 90.48% of the positive samples whereas the lateral flow-based test detected 30.95% (**Table 20**). No false positive samples were detected by either test (**Table 20**).

Table 20 | Comparison with Lateral Flow-Based Flu Test

		QFlu Test		**Lateral Flow Test**	
		Pos	Neg	Pos	Neg
RT-PCR	Pos (N=42)	38	4	13	29
	Neg (N=55)	0	55	0	55
Sensitivity		90.48%		30.95%	
Specificity		100%		100%	

X. WARNING AND PRECAUTIONS

1. Specimens in 1X Q Sample Buffer should NOT be frozen prior to testing.
2. Specimens should not be transported under extreme adverse temperature conditions. Transport should be carried out within a temperature range of 0°C (32°F) to 30°C (86°F).
3. Reagents should not be used past their expiration dates.
4. All clinical specimens and materials used to collect these specimens should be considered potentially infectious and handled accordingly. Dispose of all materials by placing in 0.5% sodium hypochlorite (1:10 dilution of household bleach).
5. The assay should be performed at 20 °C to 37 °C.
6. Samples collected in Q-Sample buffer cannot be used for culture as the Q-Sample Buffer inactivates the virus.

XI. LIMITATIONS

1. Influenza C is not detected with the QFlu Test because it does not possess a neuraminidase enzyme. Identification of Influenza C must be determined by an alternative method such as culture confirmation with monoclonal antibodies.

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XIII. ORDER INFORMATION

Item	Catalog No.
QFlu Combo Test Kit (20 Tests/Kit)	20K500
2X Q-Sample Buffer (30 mL/bottle)	30K051D
Nasal Pharyngeal Swabs (20/pkg)*	20K52
Helios 200 Analyzer	01H200

*May be purchased from alternate suppliers.

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