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Rapid, simple influenza RNA extraction from nasopharyngeal samples

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SUMMARY

This report describes the development and pre-clinical testing of a new, random-access RNA sample preparation system (TruTip) for nasopharyngeal samples. The system is based on a monolithic, porous nucleic acid binding matrix embedded within an aerosol-resistant pipette tip and can be operated with single or multi-channel pipettors. Equivalent extraction efficiencies were obtained between automated QIAcube and manual TruTip methods at 10^6 gene copies influenza A per mL nasopharyngeal aspirate. Influenza A and B amended into nasopharyngeal swabs (in viral transport medium) were detected by real-time RT-PCR at approximately 745 and 370 gene copies per extraction, respectively. RNA extraction efficiency in nasopharyngeal swabs was also comparable to that obtained on an automated QIAcube instrument over a range of input concentrations; the correlation between threshold cycles (or nucleic acid recovery) for TruTip and QIAcube-purified RNA was $R^2 > 0.99$. Preclinical testing of TruTip on blinded nasopharyngeal swab samples resulted in 98% detection accuracy relative to a clinically validated easyMAG extraction method. The physical properties of the TruTip binding matrix and ability to customize its shape and dimensions likewise make it amenable to automation and/or fluidic integration.

Keywords

RNA; sample preparation; nasopharyngeal swab; nasopharyngeal aspirate; automation

1. INTRODUCTION

Influenza viruses continue to be a significant long-term public health concern because of their genetic mutability, rapid transmission, and ability to move from species to species. Few

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drugs are approved by the US Food and Drug Administration (FDA) for treating influenza infections, and successful treatment depends on administering these drugs within the first 48 h of illness (Couch, 2000). To ensure appropriate use of antiviral drugs, a near-patient diagnostic test is therefore highly desirable (Barenfanger et al., 2000). In addition, there is a growing interest in deploying rapid detection methods in resource-limited settings (Ghosh and Vogt; Hanvoravongchai et al., 2010; Kubo et al., 2010; Ortiz et al., 2009; Oshitani, Kamigaki, and Suzuki, 2008) and expanding non-human influenza surveillance activities (e.g. (Antarasena et al., 2007; Samaan et al., 2011)).

Nucleic acid tests involving target amplification are becoming more prevalent for influenza diagnostics and surveillance (Abe et al., 2011; Bolotin et al., 2009; Habib-Bein et al., 2003; Han et al., 2008; Huber et al., 2011; Létant et al., 2007; Pachucki, Khurshid, and Nawrocki, 2004; Shisong et al., 2011; Wenzel et al., 2010; Wu et al., 2010; Xu et al., 2010), in part because of their excellent sensitivity and relatively rapid turn-around times. In many cases, these molecular detection methods are paired with automated (robotic) sample preparation technology (Agüero et al., 2007; Bolotin et al., 2009; Tewari et al., 2007), typically to increase throughput in a reference laboratory setting. Even for many upcoming molecular point-of-care devices, however, there is continued reliance upon external nucleic acid extraction methods that require some form of instrumentation (Wu et al., 2010), limiting the deployment potential of the test. Alternatively, some influenza tests utilize unprocessed nasopharyngeal samples (e.g., (Regan et al., 2010)), but limits of detection may be compromised (Létant et al., 2007) because there is either no concentration step, viral nucleic acids become inextricably bound to the sample matrix itself (and unavailable for amplification), or substances in the matrix inhibit the amplification reaction.

For molecular methods to be readily utilized in point-of-use or limited resource settings, there is a need for simple and cost-effective sample preparation systems that are consistent with the user's infrastructure, level of training, and sample throughput. The objectives of this study were therefore to develop a sample preparation system (TruTip) and methodology that only requires a pipettor (single- or multi-channel) and single tip per sample to operate, optimize a protocol for influenza RNA extraction and purification from nasopharyngeal aspirate and nasopharyngeal swabs (in viral transport media), and test the system's efficacy on a blinded set of clinical nasopharyngeal swab specimens.

2. MATERIALS AND METHODS

2.1 TruTip construction

The TruTip itself contains a highly porous, rigid, monolithic, 2 mm thick silica binding matrix of defined (yet adaptable) geometry and pore size (Figure 1). Of importance for nasopharyngeal samples, the TruTip matrix offers less fluidic impedance than a silica membrane, and combined with bi-directional flow, the system can achieve rapid extraction of nucleic acids from larger volumes than typically used in either spin-filter or bead-based sample preparation systems. This study utilized 2 mL SPT tips for single-channel sample preparation with a Rainin EDP3+ Electronic LTS pipette, and 1 mL SPT tips for a Rainin EDP3 LTS 8-channel electronic pipette (100–1200 µL).

2.2 Positive controls

Viruses used for these studies included influenza A/Hong Kong/8/68 (H3N2), influenza A/New Jersey/8/76 (H1N1), influenza A/New York/1669/2009(H1N1), influenza B/Hong Kong/5/72, influenza B/Taiwan/2/62 and a 2008 influenza B isolate from New York State. New York strains were isolated from human respiratory samples that were submitted to the reference and surveillance program at the Laboratory of Viral Diseases, Wadsworth Center, New York State Department of Health (Albany, New York). Viruses were propagated in

primary rhesus monkey kidney (pRhMK) cells as per standard procedures (Diagnostic Hybrids Inc., Athens, OH). Viral RNA was isolated from 60 μL of cultured virus using a QIAamp Viral RNA kit on the automated QIAcube, as per the manufacturer's instructions (Qiagen, Valencia, CA). Viral RNA was eluted in 60 μL total volume and 10-fold serially diluted in RNA Storage Solution (Applied Biosystems/Ambion, Austin, TX) to achieve RNA dilutions from 0 to 1:1000.

Influenza RNA copy number in each dilution was determined using single-plex, real-time reverse transcriptase TaqMan assays designed for the universal detection of influenza A matrix (M) gene and influenza B non-structural (NS) gene, as described previously (Ghedini et al., 2009). Both TaqMan assays are validated for diagnostic use. Duplicate reactions were performed at each RNA dilution level. RNA standard curves were generated from influenza M and NS RNA transcripts that had been previously quantified by UV spectrophotometry. The calculated gene copy (gc) numbers for each dilution (and replicate) were averaged to arrive at a viral stock RNA concentration (in RNA gc mL^{-1}).

In order to measure extraction efficiency, quantified green fluorescent protein (GFP) RNA transcripts were amended into nasopharyngeal swab samples prior to TruTip extraction. Transcripts were created from GFP-containing plasmid pTU65 (Chalfie et al., 1994) and quantified by real-time RT-PCR using previously described primers and probes (Tavakoli et al., 2007).

2.3 Clinical samples

Nasopharyngeal aspirate was obtained from routine clinical sampling at Little Company of Mary Hospital. De-identified, residual portions of specimens were stored frozen at -20°C until use. Nasopharyngeal swab specimens (in viral transport medium) were obtained from reference and surveillance samples submitted to Wadsworth Center and tested for influenza A and B presence using NucliSENS® easyMAG® nucleic acid extraction (bioMérieux, Durham, NC) and real-time RT-PCR assays that are approved by the New York State Department of Health for clinical use. Leftover specimens were stored frozen at -70°C until use.

Influenza-negative nasopharyngeal specimens (nasopharyngeal aspirate, or nasopharyngeal swabs in viral transport medium) were pooled and used as the sample matrix for TruTip protocol development, optimization experiments, and QIAcube comparison studies. Virus-amended matrix was serially diluted, dispensed into multiple aliquots, and frozen at -70°C . Each frozen sample was thawed only once, immediately before extraction.

For pre-clinical testing, 48 decoded specimens (32 influenza A - positive, 10 influenza B - positive, and 6 influenza - negative) were sent to Akonni Biosystems as a blinded panel for pre-clinical evaluation of TruTip extraction efficacy. Pre-clinical TruTip extractions and real-time PCR tests were also duplicated at the Laboratory of Viral Diseases as an independent verification and repeatability test (data not shown).

2.4 TruTip nucleic acid purification

A baseline TruTip extraction protocol was first developed using amended nasopharyngeal aspirate samples before applying the TruTip protocol to nasopharyngeal swabs (not shown). The optimized protocol included taking either 250 μL diluted nasopharyngeal aspirate (100 μL nasopharyngeal aspirate + 150 μL DEPC-treated H_2O) or 250 μL nasopharyngeal swab (in viral transport medium) into 375 μL of a concentrated guanidine/sodium acetate lysis buffer. Then, 375 μL of 95% ethanol was added to the sample and mixed thoroughly. For analytical studies (only), 4.4×10^4 gc of GFP transcript was added to each sample as an internal extraction and RT-PCR inhibition control. Thereafter, a Rainin EDP3+ Electronic

LTS pipette fitted with a 2 mL SPT TruTip was used to aspirate and dispense the lysed sample seven times (7 cycles) through the TruTip to bind the RNA to the TruTip matrix. The tip was then washed with 1 mL of a guanidium/sodium acetate/ethanol wash buffer for 5 cycles, followed by 1 mL of ethanol/acetone wash buffer for 5 cycles. The TruTip matrix was air-dried by cycling the pipettor 15 times in an empty tube. Finally, purified RNA was eluted from the TruTip matrix by cycling 100 μ L of an RNase-free Tris-HCl elution buffer five times through the matrix.

Extraction efficacy and reproducibility studies utilized 250 μ L aliquots of sample that were prepared by diluting influenza A- or B-amended nasopharyngeal swab in sample matrix, and then amending with 4.4×10^4 gc of GFP transcript. Amended samples were extracted and eluted into 100 μ L on three different days. Quantitative, real-time RT-PCR was performed in duplicate on all sample extracts. The extraction efficacy was determined as the lowest concentration of input viral RNA detected over all replicates across three days of extraction.

The Qiagen QIAcube served as the reference RNA extraction method for all studies, and all QIAcube samples were amended with GFP transcript as described above. In order to compare TruTip extraction efficiency to the QIAcube, 125 μ L sample and 50 μ L elution volumes were used on the QIAcube to match the volume ratios associated with TruTip extraction volumes (250 μ L sample and 100 μ L elution volumes). That is, the QIAcube is not able to process a 250 μ L sample, because the extraction vessel can only hold 2 mL whereas the total QIAcube volume of required sample + lysis buffer + ethanol would equal 2.45 mL (if performed as per the manufacturer's instructions). QIAcube samples were therefore amended with half the RNA (gene copies) as the TruTip samples to maintain a constant target nucleic acid concentration between the two extraction systems. Whether or not the 2-fold volume difference between QIAcube and TruTip had an effect on measured extraction efficiencies was not explicitly tested in this study.

2.5 Multi-channel TruTip

Multi-channel TruTip experiments utilized 1 mL SPT TruTips and the same cycling procedure as described above, except the elution volume was 75 μ L rather than 100 μ L.

2.6 RT-PCR detection

Influenza RNA was detected by real-time RT-PCR on either a LightCycler 480 (Roche, Indianapolis, IN) or Stratagene (now Agilent Technologies, Santa Clara, CA) MxP3000 thermal cycler. Regardless of the thermal cycling instrument, 5 μ L purified RNA template was amplified in duplicate (or triplicate) in a 25 μ L reaction volume using the qScript One-Step qRT-PCR kit (Quanta BioSciences, Gaithersburg, MD). Master mix components included 400 nM each primer and 325 nM internal probe for influenza A detection, and 750 nM each primer and 250 nM internal probe for influenza B detection. Cycling conditions for both influenza A and B real-time RT-PCR assays were 48°C for 20 min; 95°C for 5 min; and 45 cycles of [95°C for 15 sec, 55°C for 45 sec]. Recovered gene copy numbers were calculated relative to influenza M and NS standard curves as described above, and used to calculate a % recovery.

Cycling conditions for the GFP RNA assay were 48°C for 30 min; 95°C for 10 min; and 45 cycles of [95°C for 15 sec, 60°C for 1 min]. Recovered gene copy numbers were calculated relative to a GFP standard curve, and used to calculate a % recovery. Standard curves (for M, NS or GFP RNA targets) were generated for every RT-PCR run, with R^2 values > 0.99 over the range of 10 to 10^6 RNA copies per reaction (not shown).

3 RESULTS

3.1 RNA purification from nasopharyngeal aspirate

Nasopharyngeal aspirate was used as the specimen matrix for developing basic TruTip manufacturing and operating parameters. Preliminary fluidic experiments with nasopharyngeal aspirate samples demonstrated that sample liquefaction in lysis/binding buffer was sufficient to perfuse unprocessed nasopharyngeal aspirate over the TruTip matrix, where initial sample input volumes were 500 μL nasopharyngeal aspirate and 500 μL lysis/binding buffer (not shown). Extraction of 10^6 influenza A gc mL^{-1} nasopharyngeal aspirate ($n = 6$ per day) on two replicate days resulted in average C_t values of 31.08 ± 0.06 and 31.98 ± 0.06 for TruTip, and 31.08 ± 0.14 and 31.47 ± 0.15 for QIAcube. Given positive RT-PCR detection at 500 μL nasopharyngeal aspirate sample input and equivalent performance between QIAcube and TruTip methods, we reduced sample volumes in a step-wise fashion to arrive at the protocol described in the Methods.

3.2 RNA purification from nasopharyngeal swabs

Having developed a basic operating protocol for influenza RNA recovery from nasopharyngeal aspirate, we focused optimization efforts on nasopharyngeal swabs (in viral transport medium). Influenza A/New York/1669/2009(H1N1) and a 2008 influenza B virus (both isolated and propagated in the Laboratory of Viral Diseases) were used as material to amend into the pooled influenza-negative nasopharyngeal swabs. Amended nasopharyngeal swab samples were divided equally into 4 tubes, with an individual tube tested in duplicate at each dilution level (250 μL total sample volume) on three consecutive days. The fourth tube was processed on the QIAcube as a reference. The average TruTip processing time on the single-channel pipettor was approximately 7 minutes per sample. The sample processing speed is primarily related to the speed at which the crude lysate can be perfused through the TruTip binding matrix by the Rainin electronic pipettor, and the total number of cycles per step.

Average C_t values and recovered RNA (in gene copies) from real-time RT-PCR assays and relationship to the QIAcube reference method for nasopharyngeal swabs are shown in Table 1. TruTip extraction efficacy over all three days was 745 gene copies per extraction for influenza A and 370 gene copies per reaction for influenza B, equivalent to that obtained by QIAcube. The Pearson correlation between average TruTip and average QIAcube C_t values was $R^2 = 0.99$ for influenza A and influenza B over all positive dilutions and replicates.

All extracted samples were also amended with 4.4×10^4 GFP RNA transcript copies prior to purification on either TruTip or QIAcube as a means to detect PCR inhibition and evaluate RNA extraction efficiency from the lysed sample. The average C_t value for GFP transcripts was 30.84 ± 0.43 for TruTip and 30.43 ± 0.25 for QIAcube. For 100% extraction efficiency and the volumetrics of the TruTip procedure, we would expect to recover 2.2×10^3 GFP gene copies per extraction. From the GFP internal control, then, the measured RNA extraction efficiency was 22.7% for TruTip and 23.9% for QIAcube. From Table 1 data, the average influenza A and B RNA extraction efficiency within the central, linear portion of the dilution series (10^6 to 10^3 gene copies) was 39% and 69% for TruTip, and 50% and 81% for QIAcube, respectively. These results demonstrate efficient and repeatable TruTip RNA extraction from nasopharyngeal swabs in viral transport medium, with performance characteristics comparable to the automated QIAcube system and an estimated extraction efficacy of $10^2 - 10^3$ RNA gene copies per extraction (or $0.1 - 1$ TCID₅₀, assuming 1000 virions per TCID₅₀; (Chan et al., 2009)).

3.3 Multi-channel TruTip extractions

Extraction efficacies for the multi-channel TruTip extractions using influenza A RNA are shown in Table 2. In this case, 8 samples were processed simultaneously within 7 minutes (< 1 min per sample). The correlation coefficient between the real-time RT-PCR C_t and $\log_{10}[\text{gc mL}^{-1}]$ was $R^2 = 0.997$ with an extraction efficacy of 745 input gene copies per extraction.

Repeatability of multi-channel TruTip extraction is shown in Table 3, with results consistent with the single-channel format. In this case, repeatability at 10^2 gc per extraction was not tested given that the single-channel efficacy was defined as 10^3 gc per extraction (i.e. positive detection in 6 of 6 replicates instead of 5 of 6 replicates).

3.4 Blinded clinical samples

The optimized nasopharyngeal swab extraction protocol was applied to 48 blinded clinical nasopharyngeal swabs in viral transport medium. Typing of these blinded samples with the clinically validated real-time RT-PCR assays on a Roche 480 thermal cycler resulted in 98% accuracy (Table 4) with only one false negative result (sample NYS-47; low titer of influenza B). The blinded samples were also re-tested at Wadsworth using TruTip extraction and a Stratagene MxP3000 thermal cycler, with equivalent results (not shown). These data indicate that the TruTip nasopharyngeal swab extraction method is repeatable across test sites and users, generates high-quality viral RNA that can be amplified by different thermal cyclers, and yields test results that are comparable to those obtained with a clinically validated bioMérieux NucliSENS easyMAG nucleic acid extraction system.

4 DISCUSSION

Despite continued advances in influenza diagnostics and point-of-care systems, the extent to which molecular diagnostics are utilized beyond reference laboratory environments continues to be limited. Reasons for limited deployment or access vary depending upon the intended use and user community, but include practical considerations such as system and consumable cost; test complexity; sample volume and fluidic limitations; throughput; sample acquisition, storage and/or shipment constraints; and flexibility of the underlying technology platform. Therefore, while integrated influenza diagnostic tests are either commercially available or in development (e.g. (Jenny et al., 2010; Xu et al., 2010)), there is a continued need to provide a robust, simple, low-cost and random-access sample preparation method that has the potential to bring molecular detection systems out of a reference laboratory and closer to the point of use. The results of this study show that a very simple, pipette-operated sample preparation technology (TruTip) is as effective as clinically validated QIAcube or easyMAG automated sample preparation systems for extracting and purifying influenza RNA from nasopharyngeal samples. Whether or not volumetric differences between TruTip and automated sample preparation systems have an effect on measured extraction efficiency was not explicitly tested in this study. However, because TruTip only requires a pipette to operate and deliver PCR-quality RNA within 7 min, it therefore provides a means to bring nucleic acid sample preparation into resource-limited settings. At the same time, the TruTip sample preparation scheme is also very adaptable to robotic liquid handling systems and a higher-throughput operating environment.

The TruTip protocol described in this study should be considered a reference or starting point for nasopharyngeal samples, especially samples like nasopharyngeal aspirate. That is, every nasopharyngeal aspirate or nasopharyngeal swab sample is unique and will differ from the next in viscosity, particulates, viral load and background microflora. Sample NYS-47 (Table 4), for example, might have been detectable under a more rigorous set of binding/elution cycles. Successful influenza RNA extraction may therefore, at times, require more

vigorous up-front sample homogenization or lysis methods, or additional binding/washing/elution cycles to separate influenza RNA from the sample matrix.

The significance of these results, however, extends beyond influenza testing and diagnostics. That is, the TruTip binding matrix itself solves some very important sample preparation technical issues, especially for continued development of integrated or fully automated systems. For example, the binding matrix offers a lower fluidic impedance than membranes (e.g. as in a spin-filter or GeneXpert cartridge), as illustrated by the ability to process both nasopharyngeal aspirate and nasopharyngeal swab through the same matrix. Bi-directional flow through the TruTip accelerates nucleic acid binding kinetics relative to static (e.g. bead-based) techniques and, more importantly, is amenable to relatively large sample volumes. The latter feature is especially relevant for closing the sample-to-detector volume gap. Finally, the binding matrix itself can be manufactured with different geometries (shapes) and pore sizes to fit within commercially available pipette tips (to 5 mL) or customized microfluidic architectures. We therefore anticipate that the basic sample preparation principle, system and chemistry described here can accelerate the development and deployment of molecular diagnostic devices beyond reference laboratories.

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Highlights

- A single-tip RNA sample preparation system is described for NPA and NPS in VTM.
- The system is designed for lower-resource or random access settings.
- Input viral loads as low as 10^2 RNA gene copies per extraction could be detected by real-time RT-PCR.
- Extraction efficiency was equivalent to a QIAcube automated method.
- TruTip resolves key issues for automated or integrated sample preparation.

Figure 1. Basic TruTip construction and operation. The 2 mm thick binding matrix is embedded in an aerosol-barrier pipette tip. Fluid flow is bi-directional through the matrix. The shape and pore size of the binding matrix can be tailored to fit different tip sizes.

Table 1

Average Influenza RNA extraction efficiency in C_t values and gene copies (gc).

	TruTip Extraction ^a				QIAcube Extraction ^b			
	Input (gc)	Average C _t ± StDev	Recovered RNA (gc)	% Recovery	Input (gc)	Average C _t	Recovered RNA (gc)	% Recovery
Influenza A	7.45 × 10 ⁷	21.24 ± 0.35	3.00 × 10 ⁷	40	3.72 × 10 ⁷	20.80	2.15 × 10 ⁷	58
	7.45 × 10 ⁶	24.57±0.17	3.09 × 10 ⁶	41	3.72 × 10 ⁶	24.31	1.97 × 10 ⁶	53
	7.45 × 10 ⁵	28.07 ± 0.22	2.87 × 10 ⁵	39	3.72 × 10 ⁵	27.74	1.89 × 10 ⁵	51
	7.45 × 10 ⁴	31.51 ± 0.15	2.77 × 10 ⁴	37	3.72 × 10 ⁴	31.13	1.88 × 10 ⁴	50
	7.45 × 10 ³	34.82 ± 0.30	2.95 × 10 ³	40	3.72 × 10 ³	34.64	1.72 × 10 ³	46
	7.45 × 10 ²	39.15 ± 2.00	2.38 × 10 ²	32	3.72 × 10 ²	38.73	1.48 × 10 ²	40
	7.45 × 10 ¹	NC	NC	NC	3.72 × 10 ¹	ND	NC	NC
Average % recovery ^c =					39	Average % recovery ^c =		
Influenza B	3.70 × 10 ⁸	17.53 ± 0.20	2.38 × 10 ⁸	64	1.85 × 10 ⁸	17.41	1.86 × 10 ⁸	101
	3.70 × 10 ⁷	20.69 ± 0.18	2.70 × 10 ⁷	73	1.85 × 10 ⁷	20.53	2.09 × 10 ⁷	113
	3.70 × 10 ⁶	24.02 ± 0.22	2.85 × 10 ⁶	77	1.85 × 10 ⁶	24.06	1.74 × 10 ⁶	94
	3.70 × 10 ⁵	27.67 ± 0.22	2.37 × 10 ⁵	64	1.85 × 10 ⁵	27.44	1.63 × 10 ⁵	88
	3.70 × 10 ⁴	31.04 ± 0.44	2.40 × 10 ⁴	65	1.85 × 10 ⁴	30.90	1.44 × 10 ⁴	78
	3.70 × 10 ³	34.41 ± 0.29	2.55 × 10 ³	69	1.85 × 10 ³	34.47	1.18 × 10 ³	64
	3.70 × 10 ²	37.34 ± 0.99	3.57 × 10 ²	96	1.85 × 10 ²	38.04	9.75 × 10 ¹	53
Average % recovery ^c =					69	Average % recovery ^c =		
						81		

^aValues are the average of two replicates on each of three days (n=6) with an input volume of 250 μ L and elution volume of 100 μ L.

^bValues are the average of n=2 replicates only, with an input volume of 125 μ L and elution volume of 50 μ L. Standard deviations are therefore not provided for the average C_t values.

^c Average % recovery is calculated across target concentrations corresponding to the central, linear portion of the dilution curves (10^3 through 10^6 input gene copies, inclusive).

NC = Not calculated.

ND = Not detected.

Table 2

Multi-channel TruTip extraction efficacy for influenza A RNA.

Input (gc)	Average C_t ($\pm$ StDev)	Average Recovery (gc)
7.45×10^6	25.71 ± 0.19	1.24×10^6
7.45×10^5	28.56 ± 0.17	1.74×10^5
7.45×10^4	32.13 ± 0.45	1.60×10^4
7.45×10^3	35.84 ± 0.57	1.32×10^3
7.45×10^2	38.50 ± 0.86	2.01×10^2
7.45×10^1	Not Detected	Not Detected

Table 3

Inter-run repeatability of multi-channel TruTip extractions with influenza A RNA.

a Average TruTip Recovery in Gene Copies (and C_t)

Input (gc)	Day 1	Day 2	Day 3	Day 4	Average ^b
7.45 × 10 ⁵	3.61 × 10 ⁵ (28.08)	1.74 × 10 ⁵ (28.56)	—	—	2.80 × 10 ⁵ (28.29 ± 0.42)
7.45 × 10 ⁴	3.80 × 10 ⁴ (31.39)	3.35 × 10 ⁴ (31.28)	2.26 × 10 ⁴ (31.58)	1.60 × 10 ⁴ (32.13)	2.85 × 10 ⁴ (31.60 ± 0.44)
7.45 × 10 ³	2.98 × 10 ³ (35.18)	3.34 × 10 ³ (34.18)	2.62 × 10 ³ (34.56)	1.32 × 10 ³ (35.84)	2.56 × 10 ³ (34.94 ± 0.75)
7.45 × 10 ²	Not tested				

^a Average of three replicates per day.^b Standard deviations are provided for C_t values.

Table 4

Real-time, RT-PCR test results for TruTip-processed, blinded nasopharyngeal swab clinical specimens.

Sample	FluA Results		FluB Results		Answer ^d
	C _t	Call	C _t	Call	
NYS-3	27.46	+	-	-	pdmH1
NYS-4	35.79	+	-	-	pdmH1
NYS-5		-	-	-	Negative
NYS-6	30.95	+	-	-	pdmH1
NYS-7	33.98	+	-	-	pdmH1
NYS-8	33.45	+	-	-	pdmH1
NYS-9	30.35	+	-	-	pdmH1
NYS-10		-	-	-	Negative
NYS-11	33.14	+	-	-	pdmH1
NYS-12	36.82	+	-	-	pdmH1
NYS-13	23.24	+	-	-	pdmH1
NYS-14	36.73	+	-	-	pdmH1
NYS-15	35.77	+	-	-	H1N1
NYS-16	34.99	+	-	-	H3N2
NYS-17	30.19	+	-	-	H3N2
NYS-18		-	30.19	+	FluB
NYS-19	34.38	+	-	-	H1N1
NYS-20	28.06	+	-	-	H3N2
NYS-21		-	31.64	+	FluB
NYS-22		-	-	-	Negative
NYS-23	28.59	+	-	-	H1N1
NYS-24	29.99	+	-	-	H3N2
NYS-25		-	37.13	+	FluB
NYS-26	36.04	+	-	-	H1N1
NYS-27	36.85	+	-	-	H1N1
NYS-28		-	-	-	Negative
NYS-29		-	-	-	Negative
NYS-30	35.68	+	-	-	pdmH1

Sample	FluA Results		FluB Results		Answer ^a
	C _t	Call	C _t	Call	
NYS-31		-	36.32	+	FluB
NYS-32	28.73	+		-	H1N1
NYS-33	31.87	+		-	H3N2
NYS-34	24.97	+		-	H3N2
NYS-35		-	28.50	+	FluB
NYS-36	29.08	+		-	H1N1
NYS-37		-		-	Negative
NYS-38	26.19	+		-	H3N2
NYS-39	28.75	+		-	H1N1
NYS-40		-	35.00	+	FluB
NYS-41	25.70	+		-	H1N1
NYS-42		-	35.04	+	FluB
NYS-43	27.76	+		-	H1N1
NYS-44		-	35.58	+	FluB
NYS-45	29.30	+		-	H3N2
NYS-46 ^b		-	36.49	+	FluB
NYS-47 ^c		-		-	FluB
NYS-48	25.83	+		-	H3N2
NYS-49	33.98	+		-	pdmH1
NYS-50	32.41	+		-	H3N2

^aThe answer key is derived from clinically-validated NucliSENS easyMAG extraction method and real-time RT-PCR assays.

^bThe original RT-PCR result was negative, but positive upon re-test.

^cFalse negative result.

pdmH1 = pandemic influenza H1.