Applications

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Rapid, Automated Extraction and Purification of Influenza RNA from Nasopharyngeal Samples using the Akonni TruTip™ Extraction System on the Eppendorf ep*Motion*® 5070

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Abstract

Akonni Biosystems has developed the simple and reproducible TruTip automated extraction method to isolate viral RNA from nasopharyngeal samples on the Eppendorf ep*Motion* 5070. Flu-negative nasopharyngeal aspirate (NPA) samples were pooled and used as background for spike-recovery experiments on dilutions of intact influenza A (FluA) and influenza B (FluB) virions, with detection limits measured by real-time RT-PCR assays. FluA spiked into NPA was reproducibly extracted from five different NPA samples at 10_3, 10_2 and 10_1 gc µl$_{-1}$, and FluB at 10_3 and 10_2 gc µl$_{-1}$, consistent with protocol volumetrics and anticipated RT-PCR detection limits. Reproducibility studies on the automated system showed similar precision to the single channel electronic pipette, with low standard deviations and absence of cross-contamination.

Introduction

Major disease outbreaks are associated with the circulation of influenza virus Type A and B in human populations. Infection with Influenza B virus is usually milder than infection with Influenza A virus, but both agents are significant causes of upper respiratory illness. Clinical research in understanding and treating influenza starts with isolation of the RNA. The Akonni Biosystems TruTip Extraction Kits offer a competitive solution for fast and efficient isolation of Influenza A and B viral RNA from challenging clinical sample types without vacuum filtration or centrifugation. The technology uses a porous binding matrix embedded in a pipette tip (TruTip) and chaotropic salt chemistry. The purified sample is free of inhibitors and contaminants and ready for downstream detection via RT-PCR. Automation of the extraction process on the ep*Motion* 5070 offers a simple and low cost solution for all throughput needs. Samples are processed in multiples of eight with eight, sixteen or twenty-four samples processed in 16, 28 and 40 minutes, respectively. All reagent transfer steps are included in the method with little additional set-up time required. Herein we demonstrate the high precision and detection range of the automated extraction system in processing nasopharyngeal aspirate (NPA) samples spiked with Influenza A (FluA) or Influenza B (FluB).

Materials and methods

Eppendorf ep*Motion* 5070 equipped as follows:

- Dispensing tool TM1000-8
- Reservoir rack
- Waste tub

Eppendorf consumables:

- Reagent reservoirs: 30 ml
- Sample plate: Deepwell plate 96/2,000 µl
- epT.I.P.S. *Motion* Filtertips, 1,000 µl
- EPM TruTips: 1,000 µl

Akonni TruTip Extraction System:

- Influenza Lysis and Binding Buffer
- Influenza Wash Buffer 1
- Influenza Wash Buffer 2
- Influenza Elution Buffer

Pooled, de-identified nasopharyngeal aspirate spiked with Influenza A (H3N2) or Influenza B

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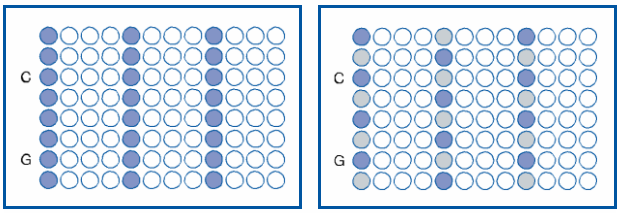

Figure 1: Sample plate layout.
A.) 24 sample set-up with all positive samples.
B.) 24 sample set-up with staggered positive and negative samples.

Reservoir Rack Position	Reservoir Contents	Reagent Prep Instructions
1	9,205 µl Lysis Buffer	None
2	9,205 µl 95% Ethanol	None
3	12,205 µl Wash Buffer 1	One time only: 95% EtOH added according to kit instructions
4	12,205 µl Wash Buffer 2	One time only: 95% EtOH added according to kit instructions
5	1,405 µl Elution Buffer	None
6	Empty (input 8,205 µl)*	Reservoir for drying
7	Empty	Unused

Table 1: Contents of the Reagent Reservoirs in the Reagent Reservoir Rack. Volumes denote minimum volume required for 24 sample program.

*A positive volume must be input into the software for position 6 to compensate for volume of air pipetted during the drying step.

Sample, Reagent and Worktable Preparation

De-identified nasal pharyngeal aspirate (100 µl) spiked with Influenza A (FluA) or B (FluB) virus was brought up to 250 µl with DEPC-treated water. Samples were dispensed into columns 1, 5 and 9 in the Sample Plate. Reagents were prepared and dispensed into 30 ml Reagent Reservoirs as described in Table 1. Labware was placed onto the ep*Motion* 5070 Worktable, as shown in Figure 2 and Table 2.

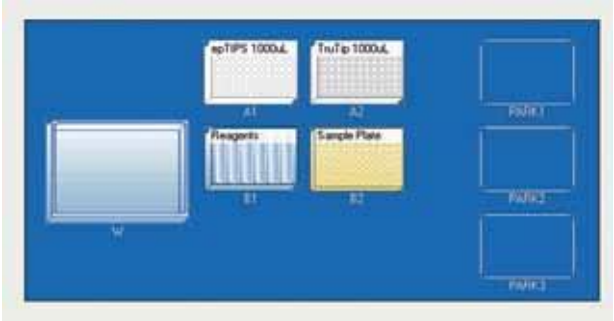

Figure 2: Screenshot from the ep*Motion* Editor showing the 5070 Worktable setup for the automated method.

Worktable Position	Labware
A1	epT.I.P.S. *Motion* Filtertips, 1,000 µl
A2	EPM TruTips, 1,000 µl
B1	Reagent Reservoir Rack
B2	Sample Plate, 96/2,000 µl

Table 2: ep*Motion* 5070 Worktable setup, details by position

Automated Method Overview

The automated method uses the Akonni TruTips to extract influenza RNA virus from nasopharyngeal aspirate or nasopharyngeal swab samples in a 24-sample format. The method begins by dispensing all reagents into the Sample Plate: 375 µl binding buffer is dispensed into wells 1, 5 and 9 and mixed with the nasopharyngeal sample; 500 µl Wash Buffer 1 is dispensed into wells 2, 6 and 10; 500 µl Wash Buffer 2 is dispensed into wells 3, 7 and 11; 50 µl Elution Buffer is dispensed into wells 4, 8 and 12. Finally, 375 µl of ethanol is added to wells 1, 5 and 9.

The dispensing tool then picks up 8 TruTips from position A2 on the Worktable and begins the extraction process for the first set of 8 samples in column 1 of the Sample Plate (Figure 1). First, the liquid in column 1 is aspirated and dispensed seven times. This step lyses the virus particles and binds the nucleic acid to the TruTip matrix. Second, the tips move to column 2 of the Sample Plate and aspirate/dispense the Wash Buffer 1 five times. Third, the tips move to column 3 of the Sample Plate and aspirate/dispense the Wash Buffer 2 five times. These two wash steps remove proteins and other contaminants from the bound nucleic acid. Fourth, the tips move to Reagent Reservoir 5 and aspirate/dispense fifteen times with air to dry the TruTip matrix. Finally, the tips move to column 4 in the Sample Plate and aspirate/dispense the Elution Buffer five times.

The extracted and purified nucleic acid is now in the elution buffer in column 4. This set of TruTips is discarded into the Waste Bin and a new set is picked up from position A2 on the Worktable.

The extraction process is repeated on the eight samples in column 5 on the Sample Plate and final extracted and purified sample is eluted into column 8. This set of TruTips is discarded into the Waste Bin and a new set is picked up from position A2 on the Worktable. The extraction process is repeated on the samples in column 9 on the Sample Plate and final extracted and purified sample is eluted into column 12. Eluted samples in columns 4, 8 and 12 are ready for analysis.

Analysis of Extracted influenza Virus

Analysis of RNA in the extracted samples in Sample Plate A and B was performed using a real-time reverse transcription and PCR (RT-PCR) on the Roche LightCycler® 480 system using Invitrogen's SuperScript® One-Step RT-PCR kit (20 µl master mix to 5 µl sample). Primers and Taqman probe sequences were developed by the CDC. All extracted samples were analyzed in duplicate. The thermal cycling conditions were as follows: 1 cycle 50 °C for 30 minutes, 1 cycle 95 °C for 2 minutes, 45 cycles of 95 °C for 15 seconds, 55 °C for 30 seconds. Fluorescence curves were analyzed using the Absorbance Quantification tool in the LightCycler software to calculate the cycle threshold value for each independent curve.

Results

Dilution Series Study

A dilution series study was performed on the TruTip-epMotion system using FluA (H3N2) and FluB spiked into five different Flu-Negative NPA samples with varying viscosity (low to high mucus content). Results are shown in Table 3 below. FluA was reproducibly detected (100%) at 10^1 gc μl^{-1}. FluB was reproducibly detected (100%) at 10^2 gc μl^{-1}, with 10^1 gc μl^{-1} approaching the detection limit of the real time RT-PCR assay.

		FluA Real-time		FluB Real-time	
Input Virus Conc.	**NPA Sample**	**Avg. Ct (n=3)**	**Std. Dev.**	**Avg. Ct (n=3)**	**Std. Dev.**
10^3 gc μl^{-1}	NPA2	33.53	0.13	30.71	0.18
	NPA3	32.60	0.09	30.93	0.06
	NPA4	33.14	0.54	31.25	0.43
	NPA5	34.35	0.25	31.81	0.29
	NPA8	33.10	0.16	30.57	0.01
	Total (n=15)	33.34	0.65	31.06	0.50
10^2 gc μl^{-1}	NPA2	37.42	0.45	34.20	0.24
	NPA3	36.83	0.46	36.75	0.74
	NPA4	37.02	0.21	36.81	0.66
	NPA5	37.27	0.51	37.54	1.15
	NPA8	36.31	0.44	36.43	0.24
	Total (n=15)	36.97	0.43	36.34	1.27
10^1 gc μl^{-1}	NPA2	38.88	0.05	39.19*	0.73
	NPA3	40.94	0.45	39.07*	1.01
	NPA4	39.97	1.29	39.03*	1.52
	NPA5	39.74	0.34	N/A	N/A
	NPA8	38.99	0.83	39.55*	0.79
	Total (n=15)	39.70	0.84	39.27	0.91
Intact virus 10^3 gc μl^{-1}		35.00		32.08	
Intact virus 10^2 gc μl^{-1}		38.44		35.33	
Intact virus 10^1 gc μl^{-1}		41.34		37.87	

Table 3: Dilution study using FluA (H3N2) and FluB spiked into five different Flu-Negative NPA samples.

*At least one real-time replicate showed negative results. A control of 5 µl intact virus for each dilution was analyzed by the real time RT-PCR assay for comparison.

Reproducibility and Cross-Contamination Study

A reproducibility study was performed by extracting the same sample from each of the 24 sample wells on a single plate. Results demonstrated high precision of this extraction method with low standard deviation using the automated system (Figure 3A). A cross-contamination study was performed by alternating positive and negative samples across the sample plate for a total of 12 positive FluA sample and 12 negative FluA samples. Results demonstrated no cross-contamination between samples when processed on the ep*Motion* 5070 automated system (Figure 3B).

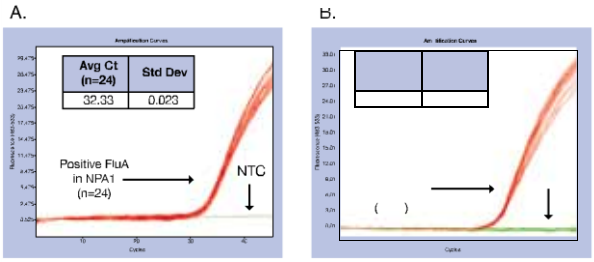

Figure 3: ep*Motion* reproducibility studies. (A) Twenty-four replicate extractions of 100 µl FluA at 10^3 gc μl^{-1} in Flu-negative NPA1 were run on a single plate according to the plate map in Figure 1 A. (B) Twelve FluA-spiked NPA7 (10^3 gc μl^{-1}) alternated with twelve FluA-negative NPA7 samples were run on a single plate according to the plate map in Figure 1 B.

Conclusion

The integration of the Akonni TruTip with the ep*Motion* 5070 resulted in a complete system for automated extraction and purification of Influenza viral RNA from nasopharyngeal aspirate samples. The flexible method is designed to process samples in multiples of eight. A full plate of 24 samples is processed in 40 minutes. All reagent transfer steps are included in the method with little set-up time required. The procedure is very easy to perform and effectively removes inhibitors to yield amplification-ready RNA. In contrast to other comparable procedures, there are no centrifugation or vacuum filtration steps required. The TruTip protocol successfully extracts and purifies RNA from FluA and FluB in NPA samples, at clinically relevant viral loads. Reproducibility studies yielded highly repeatable results at 1 x 10^3 gc μl^{-1} FluA- spiked NPA samples, with no detectable cross-contamination. We have demonstrated comparable yields to RNA isolated using other automated or manual RNA isolation procedures as well as the ability to extract and purify from nasopharyngeal swab samples in viral transport media (data not shown). This validation study confirmed that the ep*Motion* 5070 provides a reproducible and reliable method for obtaining influenza RNA from nasopharyngeal samples by use of the Akonni TruTips.

Ordering information

Description	Catalog No.
Eppendorf	
30 ml Reagent Reservoirs	960050100
Reservoir Rack	960002148
Sample Plate: Deep well plate 96/2,000 µl	0030502302
Dispensing tool TM 1,000-8	960001061
epT.I.P.S. Motion Filtertips, 1,000 µl	960050100
Akonni Biosystems	
EPM TruTip Influenza Kit (EPM Tips, 1,000 µl, 96 SPT). For use with the ep*Motion* Automated Pipetting System. Includes TruTip SPT tips and buffers for 96 extractions.	300-11120

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