



S29 REAL TIME PCR DETECTION OF INFLUENZA A VIRUS SUBTYPES USING HOMEBREW AND COMMERCIAL ASSAYS

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ABSTRACT:

The sudden appearance of pandemic influenza A H1N1 and the reported inability of rapid antigen detection devices to reveal it provided an incredible impetus to the use of nucleic acid amplification techniques to identify and characterize influenza A viruses, even in routine hospital microbiology laboratories. The CDC proposed and disseminated an ultra sensitive influenza A detection strategy. We adapted our multiplex assay that detects influenza and RSV to simultaneously detect influenza A and distinguish pandemic H1N1, seasonal H1N1, and H3N2 as well as incorporating an extraction and amplification control. Several companies have rapidly developed kits to detect this virus strain and obtained special FDA approval.

We compared the Roche MagnaPure Compact and BioMerieux EasyMag automats to extract patient samples, incorporating our RNA bacteriophage PP7 positive control in the lysis buffer added to MagnaPure samples or to the beads in the EasyMag. Extraction efficiencies were similar with less than 1% failure without operator error. The Easy Mag simultaneously extracts 24 samples in about 50 minutes and costs, in Canada, almost twice as much as a Roche MagnaPure Compact that extracts 8 samples in 30 minutes. Both have similar hands-on time and consumable costs. In our hands, the EasyMag accepted larger sample volumes (better for flocced samples in transport media) but was more prone to failure with undiluted nasopharyngeal aspirates. Using dilution series from H1N1 positive patients, we compared RT-PCR one step Master mixes from four manufacturers (Roche, Invitrogen, Bio-Rad, and Qiagen) with a Roche LC480 realtime PCR apparatus. We found that the Qiagen Quantitect Virus kit provided the most sensitive detection with the highest fluorescence amplitude with our primers and probes. With the LC480 we were able to use simultaneously 5 colors (Cyan for PP7, FAM for H1N1, HEX for seasonal H1N1, TEX615 for universal influenza A, TYE665 for H3N2).

Again with a threefold dilution series of 3 different H1N1 positive samples, one seasonal H1N1 and one H3N2 sample, we compared the CDC protocol for influenza A, our test using Fouchier's primers that distinguishes the 3 influenza subtypes with and without a positive control, the Roche RealTime Ready Influenza A/H1N1 detection set with RealTime ready RNA Virus Master kit, and the Prodesse Flu Influenza A subtyping kit on the Roche LC480 (even though Prodesse does not recommend this thermal cycler for its test). We also tested the same samples with the Seegene Influenza typing kit on a PE 9600 thermal cycler with ethidium bromide agarose gel analysis of products. Our test, the CDC test and the Seegene kit had similar sensitivities for influenza A virus. Our test and the Seegene test also effectively distinguished the subtypes, but the Seegene kit subtype specific primers were slightly less sensitive than their universal A primers, especially for H3N2. The Prodesse kit effectively distinguished the 3 subtypes, but was less sensitive as was the Roche kit which only identifies the pandemic H1N1 strain.

Primers & Probes

virus	Primer	Fragment	sequence 5' -3'
CDC	FluAcdc171F	107 bp	GACCRATCCTGTACACCTCTGAC
	FluAFouR		AGGGCATTYGGACAAAGCGTCTA
EF Flu A	FLU AM 52C	245 pb	CTTCTAACCGAGGTCGAAACG
	FluAFouR		AGGGCATTYGGACAAAGCGTCTA
Internal control PP7	PP7 2455F	183 pb	CATCGCTATGGAGCCAGAAC
	PP7 2637R		AACGTATCCGAAGCGCTAGA

Virus	Probe Name	sequence 5' -3'
FluA	FluAun209F Tx615	TEX615/TTTGTGTTACAGCCTCACCGTGCCCAAGTGA/IBRQ
H1N1	FluApan107F FAM	FAM/TGGAAAGTGTCTTTCAGGAAAGAACACAG/IBRQ
SeasH1	FluH1h111F Hex	HEX/AGA TGT ATT TGC TGG AAA GAA TAC CGA/IBRQ
H3N2	FluH3-111F665	TYE665/AGA TGT CTT TGC TGG GAA AAA CAC AGA /IBRQ
PP7	PP7 2495 Cyan	Cyan/CTGTAGGAGACACGATAAGGCGTCTCTGC/BHQ

RT-PCR EXTRACTION: EASYMAG versus MAGNAPURE COMPACT

MagnaPure Compact

Sample Prep: 0.25 ml of sample added to 465 µl Roche lysis buffer with added DTT, polyA, and internal control (RNA bacteriophage P7).

Preparation 10-15 minutes for 8 samples then 26 minutes in the robot



	InfluenzaA			InfluenzaA H1N1			InfluenzaB			Internal Control		
	mPure	EasyM	EasyM	mPure	EasyM	EasyM	mPure	EasyM	EasyM	mPure	EasyM	EasyM
	.25ml	.25ml	.5ml	.25ml	.25ml	.5ml	.25ml	.25ml	.5ml	.25ml	.25ml	.5ml
1							19.99		16.82	23.97		20.77
2							15.56	15.93	15.84	20.08	19.98	19.95
3	22.86	22.22	22.33		22.72	22.69				24.48	23.33	23.29
4	21.46		21.56	20.79		21.26				23.85		23.24
5	22.72	22.57	21.82	22.59	22.06	21.36				23.81	23.83	24.00
6	22.70	22.22	22.25	22.06	22.15	21.89				23.77	23.52	23.72
7	17.91			18.58								18.34
8	19.85	19.87	19.75	19.91	20.14	20.02				22.59	21.73	21.39
9	24.64	23.06	22.54							24.32	23.55	23.52
10	21.39	20.73	20.56	21.03	20.78	20.59				23.04	22.85	22.50

Ten nasopharyngeal aspirates containing either influenza A/H1N1 (samples 3-8,10), influenza A/H3N2 (sample 9) or influenza B (samples 1&2) were prepared from 0.25 ml or 0.5 ml aliquots. RT-PCR was performed. The crossing points are noted. Blank spaces indicate no amplification.

Mucoid nasopharyngeal samples had to be diluted in saline for the EasyMag, but the MagnaPure tolerated them better. The EasyMag accepts up to 1 ml of sample and the MagnaPure Compact only 250 µl in our protocol, so larger samples from flocced swabs could be used with the EasyMag.

EasyMag

The apparatus delivers lysis buffer into 8 chamber reaction vessels. Patient sample (<1ml) is added to each vessel, mixed, and incubated 10 min. Silica beads together with internal control (RNA bacteriophage P7) are then added to each vessel.

Preparation 15-20 minutes for 24 samples then 45 minutes in the robot



We tested 5 three fold dilutions (in a pool of Flu A negatives) of 3 clinical samples positive for H1N1, and samples positive for H3N2 and seasonal H1N1 influenza. The CDC protocol (C) detected 3 or 4 dilutions of each sample with the influenza A probe. We did not test their H1N1 protocol. Our protocols with only FluA primer and H1N1 probe (E1) or all 3 subtype probes (E4) detected most dilutions. When we added the internal control (IC) to E1, it detected FluA with similar sensitivity to the CDC. The Prodesse (P) and Roche (R) kits only detected the first dilution for 4 of the 5 samples (1 H1N1 not detected). Prodesse correctly distinguished the influenza types and Roche detected H1N1. The Seegene (S) kit detected FluA/H1N1 in almost all the dilutions but only detected the first H3N2 dilution.

Typical experiment



CONCLUSIONS and PERSPECTIVES

- It is possible to simultaneously detect the 3 common subtypes of human influenza A virus in a single PCR reaction with a positive control with our protocol, the Prodesse protocol or the Seegene kit. Indeed, multiple probes to a PCR primer pair can be added without decreasing sensitivity, but adding additional primers may reduce efficiency.
- Future studies will need to be undertaken to appreciate the clinical usefulness (necessity) of the high sensitivity that these kits can attain.
- The Prodesse kit should be tested in a PCR apparatus that the company recommends.
- In light of its great sensitivity, it would be interesting to test the Seegene kit with their detection apparatus that is more friendly to clinical laboratory technicians than the agarose gels we employed.

+ detected in the high confidence mode

± detected in high sensitivity mode or weakly visible band by Seegene .