

RT-PCR/electrospray ionization mass spectrometry approach in detection and characterization of influenza viruses

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Reverse-transcription PCR (RT-PCR) coupled with electrospray ionization mass spectrometry (ESI-MS) is a high-throughput nucleic acid-based technology that relies on the accurate measurement of the molecular weight of PCR amplicons that can be used to deduce the base counts (number of As, Gs, Cs and Ts) of DNA. These amplicons represent highly variable regions with information-rich sequences, which are flanked by broad-range primers designed based on highly conserved loci. This technology was first introduced in 2005 for microbial identification and subtyping, and was later applied to influenza virus detection and identification. The influenza RT-PCR/ESI-MS assay allows analysis of approximately 300 samples per 24 h, and aids in the characterization of influenza viruses based on their 'core' gene signatures. Notably, this assay was used to identify one of the first cases of the 2009 H1N1 pandemic viruses. One of the main advantages of the RT-PCR/ESI-MS technology is its universality and adaptability for pathogen characterization. Efforts are being made to customize the currently used influenza surveillance assay for use in the diagnosis of the H1N1 pandemic virus. In this article, we provide a summary of known applications of the RT-PCR/ESI-MS assay in the field of influenza.

KEYWORDS: core genes • H1N1 pandemic • mass spectrometry • PLEX-ID • reverse-transcription PCR/electrospray ionization mass spectrometry • seasonal influenza • T5000

Annual influenza epidemics, sporadic outbreaks of highly pathogenic avian strains and occasional pandemics continue to pose serious concerns for human health, livestock and poultry production. The 21st Century's first pandemic of influenza H1N1 is a reminder of the importance of molecular methods in the management of responses to current and future threats. A number of laboratory methods are currently in use for influenza surveillance and diagnosis, and they include serological assays [1], conventional reverse-transcription (RT)-PCR or real-time RT (rRT)-PCR assays [2–5], pyrosequencing [6] or microarrays [7,8].

The last 2 years have seen an exponential rise in the amount of research funding made available for the development of rapid diagnostic devices and methods for infectious agents of medical importance, including influenza viruses. Each of these methods has certain advantages and limitations. For instance, some allow very rapid (<15 min) detection of

influenza virus and recognition of type A versus type B but do not have subtyping capability, and some are limited to identifying one or two subtypes; other methods are more sensitive and conclusive but are more time consuming or not suited for a high-throughput format. The most commonly used molecular method for influenza diagnostics and identification is the rRT-PCR. Although sensitive, rapid and accurate, it is largely designed for characterization of the hemagglutinin (*HA*) and *M* genes. It does not allow subtyping of the neuraminidase (*NA*) and may not allow identification of emerging viruses with reassorted internal genes, such as the polymerases. The selection of which method to use is, therefore, influenced by a combination a number of factors, including fitness for purpose, sensitivity, specificity, technical ease and cost. Complete genome sequencing coupled with phylogenetic analysis remains the gold standard for absolute influenza virus identification; however, this method is low throughput,

labor intensive and expensive. One of the new molecular methods is RT-PCR/electrospray ionization (ESI) mass spectrometry (MS), which was originally designed for microbial detection and characterization [9–11], and was later applied for influenza virus characterization [12,13]. The influenza RT-PCR/ESI-MS assay is a complementing tool to the array of assays currently in use, with its own advantages and certain limitations.

Identification of influenza virus using the RT-PCR/ESI-MS assay is based on the determination of the base counts of RT-PCR amplicons, representing highly variable loci on the influenza genome [12]. However, it is important to note that in its current format, the assay does not target the *HA* and *NA* genes, and the subtypes of these surface antigens of the influenza A viruses are inferred based on the analysis of the ‘core’ genes. Such an approach is not unique to this assay, and has been utilized previously in the development of other molecular assays intended for use in influenza diagnosis and surveillance [14,15]. In those studies, HA and NA subtypes of tested viruses were deduced based solely on *M* gene recognition using a low-density microarray. Inference of subtypes is not unusual in virus detection assays, especially in case of the NA, and most of the currently used assays, including the rRT-PCR, do not subtype the NA and rely mainly on subtyping the HA to infer the NA subtype.

Nevertheless, it is important to emphasize that inference of the HA and NA subtypes based on one or more internal genes is applicable only to stable lineages. The potential occurrence of reassortment presents a serious challenge to the methods that rely on deducing HA and NA subtypes. For instance, seasonal H1N2 viruses that acquired the *HA* gene from seasonal H1N1 viruses in 2001–2002 would be recognized as typical seasonal H3N2 viruses, since the internal genes were of H3N2 lineage [16]. Similarly, if an H1N1 pandemic virus would acquire a novel surface antigen – HA, from an avian H5N1 virus – it would still be recognized as a typical H1N1 pandemic virus.

Principle of the influenza RT-PCR/ESI-MS assay & instrumentation

The principle of the influenza RT-PCR/ESI-MS assay is based on the concept of triangulation, which combines the nucleotide base-composition (BC) signatures from multiple loci on the influenza genes to generate ‘genomic prints’ that are unique and specific to different virus groups or stable genetic lineages and allow their characterization. The assay involves several important steps that include sample collection and RNA isolation, the generation of amplicons using the Influenza Surveillance Kit (Abbott Molecular, IL, USA), including the primers, desalting and purification of PCR products, acquisition of ESI-MS signals and spectral analysis, and sample identification by comparison with a database (DB) of reference viruses that include predetermined BC signatures and/or genomic prints.

To date, all published studies have been performed on the T5000 instrument; however, FIGURE 1 is a photograph presentation of the Ibis T6000 biosensor instrument (also known as the PLEX-ID; Abbott Molecular) [101], in which all post-RT-PCR steps, including data analysis, are automated. The PLEX-ID represents a redesign of the original T5000 platform, and offers several key advantages over the preceding platform. Using a novel dual-probe ESI assembly, the PLEX-ID is capable of analyzing one well with PCR product every 30 s (twice as fast as the T5000) and, thus, completes analyzing an eight-well set of individual RT-PCR products for one specimen every 4 min. The anion-exchange resin-based desalting/purification chemistry employed by the PLEX-ID is the same as that used on the T5000 [17], although it is carried out on a rotating carousel that utilizes 22 identical spin cuvette modules. This configuration reduces both liquid waste and plastic-ware usage relative to the T5000 and affords enhanced reliability. The PLEX-ID input stacker allows the user to load 15 96-well PCR plates

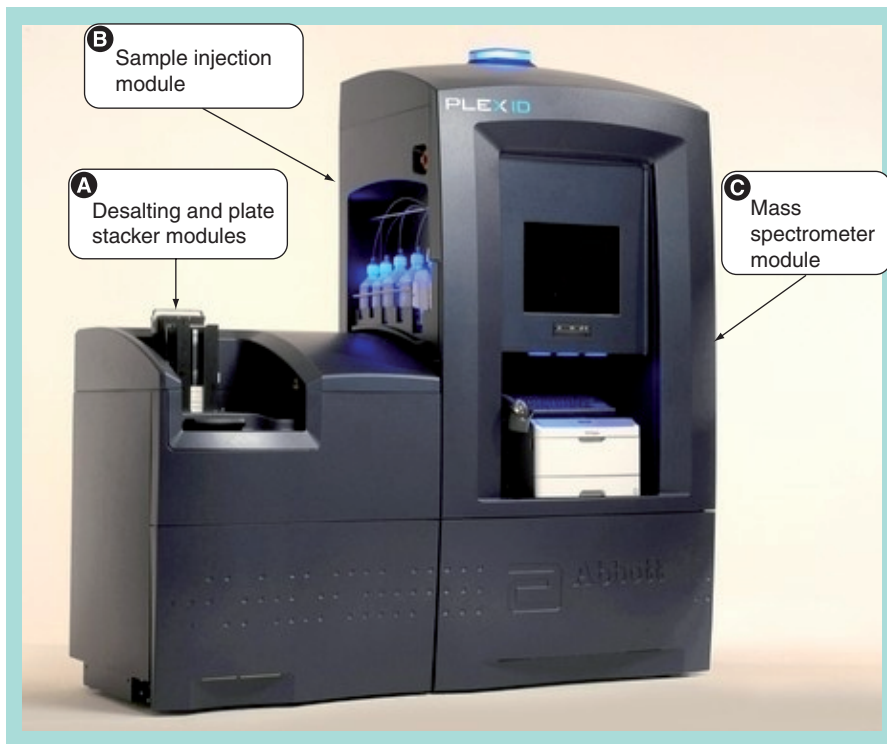


Figure 1. Ibis T6000 (PLEX-ID) universal biosensor system. PCR products are analyzed through the automated modules of the instrument. (A) First, aliquots from each reaction are desalted and purified, (B) samples are transferred and (C) injected into the mass spectrometer and their raw mass spectra are determined and postcalibrated using the high-throughput electrospray ionization mass spectrometer module. Finally, the precise base composition of each amplicon is determined, and the base-composition signatures are compared with those present in the Ibis database for sample characterization.

(accommodating up to 180 specimens analyzed for eight targets per load), and has an additional 'stat' drawer into which an assay plate can be placed for priority analysis. Noteworthy, unlike the T5000 platform, the PLEX-ID was designed and built under a rigorous US FDA-compliant design-control system, and is intended for eventual use in a clinical diagnostic capacity. At present, the PLEX-ID is a 'research-use only' device and is not cleared for diagnostics. The analytical sensitivity of the PLEX-ID platform was demonstrated to be 31–125 genome copies per PCR well [18].

Assay primer design

The primers are provided in the Influenza Surveillance Kit and were designed to target the core gene segment sequences from all influenza viruses of types A (subtypes HxNy, where x = 1–16, and y = 1–9), B and C, which are available in public domains. The gene sequences were aligned and eight amplification primer sets were chosen. The eight primer sets (TABLE 1) were designed from well-conserved regions, ranging from 101 to 129 bp and generate BC signatures that permit differentiation of the core genes of viruses that belong to stable genetic groups [12,13]. One primer pair, 2798, was designed to partially amplify PB1 from all known influenza viruses of all known origins ('pan-influenza'). Five other sets of primers, 1287 (PA), 1266 (NP), 1279 (M1), 2775 (NS1) and 2777 (NS2), were designed to amplify sequences of only

influenza viruses of type A ('pan-influenza A'). For amplification of influenza B viruses only, two pairs of primers, 1261 (PB2) and 1275 (NP; 'pan-influenza B'), were selected.

The primary constraint for primer design for this assay is the length limitation for the total PCR amplicon. For the accurate conversion of masses to base composition signatures, the amplicons have to be smaller than 150 bp. Furthermore, a multiple sequence alignment that shows regions of conservation of primer targets across multiple target sequences is necessary to achieve broad priming. Most of the other constraints are not different from other PCR design approaches. Target regions are evaluated on the basis of minimizing primer dimerization and hairpin formation, for example [10].

The primers were then tested and validated using 303 influenza samples from clinical specimens and cultured viruses [12,13]. More recently, the same assay was employed for analysis of over 750 viruses, including the H1N1 pandemic viruses [18].

RNA preparation & RT-PCR

The 96-well plate Influenza Surveillance Kit contains all the reagents required for the RT-PCR amplification. Only RNA samples, prepared as described elsewhere [12,18], were added. An example of plate layout is shown in FIGURE 2. In this format, each plate can be used to test 12 viruses. The conditions of the amplification are provided in detail elsewhere [12].

Table 1. Details of the eight primer pairs contained in the influenza reverse-transcription PCR/electrospray ionization mass spectrometry assay kit.

Primer ID	Gene segment targeted	Influenza type	Primer sequence	Amplicon length (bp)
2798	PB1	A, B, C	F: TGTCTGGAATGATGATGGGCATGTT R: TCATCAGAGGATTGGAGTCCATCCC	128
1266	NP	A	F: TACATCCAGATGTGCACTGAACTCAAACCTCA R: TCGTCAAATGCAGAGAGCACCATTCTCTCTA	111
1279	M1	A	F: TCTTGCCAGTTGTATGGGCCTCATATAC R: TGGGAGTCAGCAATCTGCTCACA	115
1287	PA	A	F: TGGGATTCCTTTCGTAGTCCGA R: TGGAGAAGTTCGGTGGGAGACTTTGGT	122
2775	NS1	A	F: TCCAGGACATACTGATGAGGATGTCAAAAATGCA R: TGCTTCCCCAAGCGAATCTCTGTA	129
2777	NS2	A	F: TGTCAAAATGCAATTGGGGTCTCATC R: TCATTACTGCTTCTCCAAGCGAATCTCTGTA	105
1261	PB2	B	F: TCCATTGTACTGGCATACTGCTTGA R: TATGAACTCAGCTGATGTTGCTCCTGC	101
1275	NP	B	F: TCCAATCATCAGACCAGCAACCCTTGC R: TCCGATATCAGCTTCACTGCTTGTGG	110

Primer pair 2798 (PB1) allows amplification of a fragment from all influenza types A, B and C. Primers targeted to influenza type A viruses are capable of amplifying all influenza A viruses but do not crossamplify B and C types of influenza. Similarly, primers designed for the B type of viruses amplify all B viruses and not those of A and C types.

F: Forward; ID: Identification; R: Reverse.

Adapted from [12].

ESI-MS run, data acquisition & analysis

Following RT-PCR amplification of the targeted sequences, samples were transferred into the Ibis T5000 or T6000 (PLEX-ID) instruments, and automated PCR product desalting/purification and ESI-MS were performed [10,17]. The mass spectrometer determines the weight of each amplicon with sufficient accuracy to allow measurement of the number of different nucleotides without ambiguity [11]. For each target, a BC signature was determined. Combinations of BC signatures from the six loci, in the case of influenza A (PB1, NP, M1, PA, NS1 and NS2), or three loci (PB1, PB2 and NP) for influenza B viruses, constitute a genomic print, and these were then compared with an existing library of genomic prints in a DB. These reference BC signatures (e.g., FIGURES 3 & 4), derived from sequences of known influenza A viruses, can be used to characterize the genes of viruses present in the samples tested. The criterion for an acceptable result using the RT-PCR/ESI-MS was set to be the detection of product in at least five of the six influenza A primer sets included in the kit [12]. In cases where one or more of the primer pairs did not amplify the targets, samples were further analyzed to determine the source of the failed amplification. The efficiency and robustness of the RT-PCR/ESI-MS assay are based on identification of the virus match ('hit') in the reference DB. This match refers to a single and/or multiple targets and a log-likelihood ratio is used to measure the goodness of this fit [10].

The RT-PCR/ESI-MS assay relies on external DBs only for naming the organisms and identifying them with the associated attributes, as defined in the reference sequences obtained from the various public domain DBs, such as Genbank or FluDB. The digital nature of the measurements made by this assay makes it capable of enhancing the existing data and, thus, provides a possibility of constantly updating the data. Theoretically, the DB could be updated as frequently as needed, although in practice, it is updated when there is a significant change in the underlying signature. For instance, the detection of the pandemic 2009H1N1 was accomplished without a DB update, although subsequent identification of this particular signature was accomplished with a DB update.

Applications

This section provides summaries of reported studies on the use of the influenza RT-PCR/ESI-MS assay. Of note, the studies reported in this article were performed at the WHO Collaborating Center for Surveillance, Epidemiology and Control of Influenza at the US CDC (GA, USA), using specimens collected in the USA and other countries, as detailed elsewhere [18], and by Ibis Biosciences, Inc., a subsidiary of Abbott Molecular (CA, USA). Details regarding the source and type of the different specimens tested at Ibis were described previously [12,13]. Furthermore, the initial validation of

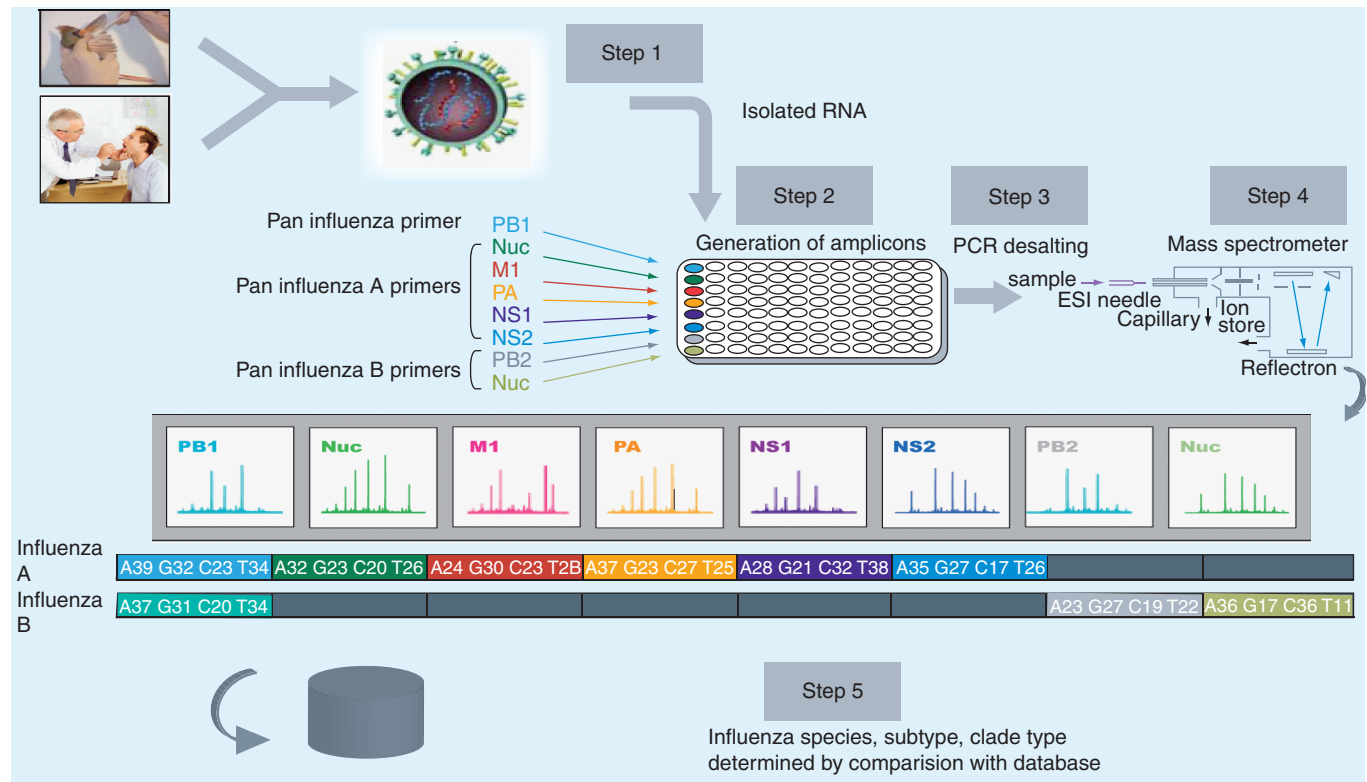


Figure 2. Principle of the influenza reverse-transcription PCR/electrospray ionization mass spectrometry assay. Viral RNA is prepared from either clinical specimens or from grown isolates (step 1). Eight primer sets (TABLE 1) are used to amplify targets from both influenza A and B virus 'core' genes (step 2). RT-PCR products are purified and desalted (step 3). Samples are then injected through the electrospray ionization needle and their spectral mass is determined (step 4). Postcalibration and spectral analyses are followed by accurate determination of the base-composition signature of each amplicon, and characterization of virus strains by comparison with viruses with predetermined 'core' gene base-composition signatures in the Ibis database (step 5).

Host	Subtype	Targeted genes					
		Pan-flu-PBI	Pan-fluA-NP	PAn-fluA-M1	Pan-fluA-PA	Pan-fluA-NSI	PAn-fluA-NS2
Human	H3N2	39 32 24 33	32 24 20 25	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
	H3N2	39 32 23 34	32 24 20 25	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
	H3N2	39 32 23 34	32 23 20 26	24 30 23 28	36 24 28 24	38 32 21 28	35 27 17 26
	H3N2	40 31 24 33	31 25 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
	H3N2	40 31 24 33	32 24 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
	H3N2	39 32 24 33	31 25 21 24	25 29 22 29	36 24 27 25	39 31 21 28	36 26 17 26
	H3N2	40 31 24 33	32 24 21 24	25 29 23 28	36 24 27 25	39 31 21 28	36 26 17 26
	H1N1	36 30 26 36	33 23 20 25	21 32 22 30	36 27 27 22	37 32 22 28	33 28 18 26
	H1N1	36 30 26 36	31 25 20 25	22 31 25 27	36 26 28 22	37 32 22 28	35 26 18 26
	H1N1	36 30 26 36	33 23 20 25	22 31 23 29	36 26 29 21	38 31 22 28	35 26 18 26
	H1N1	33 32 27 36	34 22 21 24	24 27 26 28	36 26 27 23	36 33 22 28	33 28 18 26
	H1N1	33 32 27 36	34 22 21 24	24 27 27 27	36 26 27 23	36 33 22 28	33 28 18 26
	H1N1	33 32 27 36	34 22 21 24	24 27 27 27	36 26 27 23	35 34 22 28	32 29 18 26
	2009 N1N1pdm	39 32 24 33	35 21 20 25	24 28 24 29	36 25 27 24	36 35 20 28	34 29 16 26
	2009 N1N1pdm	39 32 24 33	35 21 20 25	24 28 24 29	35 26 27 24	36 35 20 28	34 29 16 26
Swine	H1N1	39 32 26 31	33 24 20 24	25 27 25 28	34 28 28 22	35 35 21 28	33 29 17 26
	Unknown	39 32 25 32	35 22 21 23	22 28 26 29	35 27 27 23	35 36 21 27	33 30 17 25
Equine	H7N7	39 32 23 34	38 20 21 22	25 27 21 32	35 27 27 23	40 29 21 29	35 26 17 27

Figure 3. Examples of reverse-transcription PCR/electrospray ionization mass spectrometry assay-generated 'genomic prints' from human seasonal, high- and low-pathogenic influenza A viruses, swine viruses, as well as the H1N1 pandemic virus. The unique genomic prints allow characterization of the different subtypes HxNy based on the 'core' genes targeted. Each subtype (e.g., seasonal H1N1, H3N2 and H5N1) is color-coded for clarity. Within the same subtype, the different coloring patterns represent variations between the major clades (groups) of the same subtype. Seasonal human H3N2, H1N1 and the H1N1 pandemic viruses are marked in blue, green and purple boxes, respectively, in the subtype column. H: Hemagglutinin; N: Neuraminidase.

the influenza RT-PCR/ESI-MS assay was accomplished using 50 different grown influenza A virus isolates of avian, human, swine and equine origin, as well as human B viruses.

Surveillance of human & avian influenza viruses

The RT-PCR/ESI-MS assay was used to analyze over 650 clinical samples collected between 1999 and 2006 [12]. The results accurately predicted the presence of influenza viruses in approximately 240 specimens, while the remaining ones were negative. The detected viruses included A(H1N1), A(H3N2) and B. In addition, testing and the identification of 63 avian isolates, including high pathogenicity avian influenza virus H5N1 viruses, collected in Egypt and Asia, was reported by Sampath *et al.* Moreover, other viruses of swine origin were also tested and characterized [12,13].

The authors reported that virus types and subtypes inferred from the genomic prints determined based on the core genes concurred with those determined by HA inhibition (HI), rRT-PCR and conventional gene-sequencing assays, with over 97% sensitivity and 98% specificity [12]. In this study, an internal RNA control with a known number of copies was included in each of the eight target amplicon reactions. This allowed approximate quantitation of the amount of viral genome load in the samples tested.

After establishing that the RT-PCR/ESI-MS assay provided a rapid and high-throughput tool for influenza surveillance [12], it was used to analyze the different genetic lineages of the influenza type A(H3N2) viruses that circulated from 1996 to 2006. Sampath *et al.*, in their study, used H3N2 viruses with complete genome

sequences available in GenBank to construct a phylogenetic tree to enable the identification of different genetic groups [12]. The authors used a RT-PCR/ESI-MS assay to test the same set of viruses. The BC signature at each locus of the six influenza A targets analyzed for each virus was assigned a letter and the six letter sequence was termed 'lineage'. The founder (ancestor) virus was assigned the lineage 'AAAAAA' as a reference. Sequence variations from the reference BC signatures at any of the six loci are referred to by a letter other than A [12]. The alignments of the six letters (lineages) from the 104 viruses were mapped onto the phylogenetic tree obtained from the full H3N2 genome sequences. The comparative analysis demonstrated a similar pattern of distribution of lineages identified by the RT-PCR/ESI-MS assay and the genetic groups (clades) determined based on complete genome sequencing [19]. Furthermore, BC signature-derived lineages of North American H3N2 viruses from the 2005–2006 influenza season were included and the analysis revealed the predominance of the AADFAA lineage among the 2005–2006 season viruses investigated. Interestingly, the same lineage was also found to be the most predominantly circulating lineage during the previous winter in the southern hemisphere. Those findings suggested that the 2004–2005 lineage was probably the precursor of the dominant 2005–2006 lineage. These data are in accordance with the studies by Simonsen and coworkers, who used full-genome sequencing and phylogenetic analyses to describe the genesis and spread of the N-lineage of adamantane-resistant H3N2 viruses [20], which correspond to those of the AADFAA lineage, determined by the RT-PCR/ESI-MS assay [12].

Host	Subtype	Targeted genes					
		Pan-flu-PB1	Pan-fluA-NP	Pan-fluA-M1	Pan-fluA-PA	Pan-fluA-NS1	Pan-fluA-NS2
Avian species							
American black duck	H3N2	37 34 23 34	34 24 21 22	21 32 24 28	35 26 29 22	37 32 22 28	34 27 18 26
American green-winged teal	H8N4	39 32 23 34	36 22 21 22	24 28 25 28	34 27 28 23	37 32 22 28	34 27 18 26
Blue-winged teal	H1N3	37 33 24 34	35 22 22 22	23 30 25 27	35 27 28 22	40 30 20 29	35 27 16 27
Blue-winged teal	H1N4	40 31 24 33	35 22 22 22	24 28 25 28	35 26 28 23	40 30 20 29	35 27 16 27
CITE	H8N4	40 31 24 33	35 22 22 22	22 30 27 26	34 27 29 22	40 30 20 29	35 27 16 27
Mallard	H10N7	40 31 24 33	35 22 21 23	23 30 26 26	34 27 29 22	37 32 22 28	34 27 18 26
Mallard	H4N6	39 32 23 24	35 22 23 21	22 31 27 25	34 27 28 23	37 32 22 28	34 27 18 26
Mallard	H4N8	40 31 22 35	34 24 21 22	25 28 25 27	34 27 28 23	37 32 22 28	34 27 18 26
Mallard	H5N2	39 32 23 34	36 21 22 22	23 31 26 25	35 26 27 24	37 32 22 28	34 27 18 26
Mallard	H5N3	39 32 24 33	34 24 21 22	21 32 25 27	34 27 28 23	37 32 22 28	34 27 18 26
Mallard	H6N5	39 32 22 35	36 21 22 22	21 32 25 27	34 27 28 23	40 30 20 29	35 27 16 27
Mallard	H7N7	39 32 23 34	36 22 22 21	20 32 27 26	34 27 29 22	37 32 22 28	34 27 18 26
Ruddy turnstone	H10N7	40 31 24 33	33 25 21 22	23 30 25 27	35 27 27 23	38 31 22 28	34 27 18 26
Ruddy turnstone	H11N6	38 33 22 35	35 22 22 22	23 30 24 28	34 27 28 23	40 30 20 29	35 27 16 27
Ruddy turnstone	H12N4	40 31 23 34	35 23 22 21	23 30 26 26	35 26 29 22	37 32 21 29	34 27 17 27
Ruddy turnstone	H12N5	39 32 24 33	36 21 22 22	21 32 24 28	35 26 29 22	37 32 22 28	34 27 17 27
Ruddy turnstone	H2N4	39 32 24 33	35 22 22 22	23 30 24 28	35 26 29 22	37 32 22 28	34 27 17 27
Ruddy turnstone	H2N9	39 32 24 33	36 21 22 22	24 29 25 27	35 26 29 22	37 32 22 28	34 27 17 27
Ruddy turnstone	H3N8	37 34 23 34	34 24 20 23	23 30 24 28	34 27 28 23	37 32 22 28	34 27 17 27
Ruddy turnstone	H7N9	39 32 24 33	36 21 22 22	21 32 24 28	34 27 29 22	37 32 22 28	34 27 17 27
Ruddy turnstone	N9N5	38 33 23 34	37 21 20 23	24 28 23 30	35 26 28 23	37 32 22 28	34 27 17 27
Ruddy turnstone	H9N7	38 33 24 33	37 21 20 23	22 30 26 27	35 26 28 23	37 32 22 28	34 27 17 27
Teal	H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 27 17 27
Chicken	H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	38 31 21 29	35 27 16 27
Chicken	H5N1	37 32 25 34	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 28 16 27
Human	H5N1	37 32 26 33	34 23 21 23	23 29 24 29	35 25 29 23	37 32 21 29	34 28 16 27
Goose/chicken/duck/human	H5N1	37 32 26 33	34 23 21 23	23 29 24 29	35 25 29 23	38 31 21 29	35 27 16 27
Egret/chicken/duck/human	H5N1	37 32 26 33	34 23 21 23	23 29 25 28	36 24 29 23	37 32 21 29	34 28 16 27
Pintail	H5N3	38 32 26 32	34 24 23 20	23 30 25 27	34 27 29 22	40 30 20 29	35 27 16 27
Goose	H7	38 32 26 32	34 24 22 21	23 30 25 27	34 27 28 23	39 31 21 28	35 27 17 26
Shoveler	Unknown	37 33 24 34	36 23 19 23	24 29 22 30	33 27 30 22	43 30 20 26	34 28 16 27
Shoveler	H10	39 31 26 32	34 24 22 21	23 30 25 27	34 27 29 22	37 32 22 28	34 27 18 26
Shoveler	H10	38 32 26 32	35 23 22 21	24 30 26 25	35 26 29 22	37 32 22 28	34 27 18 26
Shoveler	H9N2	37 32 26 33	34 24 22 21	23 30 25 27	33 28 29 22	37 32 22 28	34 27 18 26
Teal	H10	38 32 26 32	34 24 22 21	24 29 25 27	34 27 29 22	37 32 22 28	34 27 18 26
Teal	H11	37 33 26 32	34 24 23 20	24 29 25 27	34 27 29 22	37 32 22 28	34 27 18 26
Teal	H5N2	38 32 26 32	34 24 23 20	24 29 25 27	34 27 29 22	40 30 20 29	35 27 16 27
Teal	H9	39 31 26 32	33 25 21 22	24 29 25 27	34 27 29 22	37 32 21 29	34 27 17 27

Figure 4. Examples of genomic prints determined using the RT-PCR/ESI-MS assay for influenza viruses of avian origin. Base composition signatures unique to each of the subtypes were determined as described in FIGURE 3. Virus subtypes were also color-coded for clarity, similar to FIGURE 3. Human and avian H5N1 viruses are labeled in the red box. H: Hemagglutinin; N: Neuraminidase.

During the following 2006–2007 influenza season, new antigenic variants of H3N2 viruses, which are different from the vaccine strain A/Wisconsin/67/2005, emerged. These co-circulating genetic groups included both adamantane-sensitive and -resistant viruses [21–23]. Subsequently, the usefulness of the RT-PCR/ESI-MS assay in screening a large number of H3N2 viruses to

identify major genetic lineages in circulation was assessed [18]. For this purpose, 65 viruses were analyzed and were all represented by 20 unique genomic prints [18]. The most dominant genomic prints among the adamantane-resistant viruses were AADFAA, CCDFAA and CCLFAA. Of note, the AADFAA genomic print was shared between H3N2 viruses that circulated

in prior seasons, specifically the N-lineage [12,20]. Comparison of the RT-PCR/ESI-MS results with full-genome sequencing and phylogenetic data of the same 65 viruses showed that the 20 representative genomic prints identified clustered according to the four genetic lineages (A–D) determined by phylogenetic analysis [24].

Analysis of 95 H3N2 viruses collected during the following influenza season (2008–2009), using the RT-PCR/ESI-MS assay, revealed that they all retained the genomic prints CCDFAA or CCLFAA, which are characteristic of the D lineage, with no apparent evidence of major genetic changes based on this assay. These observations were consistent with the results obtained based on phylogenetic analyses [24].

Collectively, these studies suggested that the RT-PCR/ESI-MS assay could serve as a primary screening tool to identify emerging variants among co-circulating viruses, and as a valuable tool for the rapid testing of large numbers of viruses. Such information could be valuable in decision making and for recommendations on new vaccine strain selection and M2 antiviral drug (adamantanes) usage.

Analysis of H1N1 virus clades & characterization of reassortant dual-resistant H1N1 viruses

The vast majority of recently circulating seasonal (prepandemic) H1N1 viruses are either resistant to adamantane drugs (clade 2C) or to NA inhibitors (clade 2B) [25–27]. However, the genesis and spread of dual-resistant viruses through reassortment between these two co-circulating clades are of public health concern. Indeed, a number of viruses resistant to both adamantanes and oseltamivir were detected in Asia and North America [25,26,28].

The RT-PCR/ESI-MS assay's ability to identify seasonal H1N1 viruses and the clades to which they belong and to recognize those emerging from reassortment between the two clades was assessed [18]. Viruses A/South Dakota/06/2007 (clade 2B), A/Colorado/UR06–0053/2007 (clade 2C) and A/Texas/57/2009 (reassortant 2B/2C clades) with experimentally predetermined genomic prints were used as reference strains for the identification of tested samples. In total, 37 seasonal H1N1 viruses were analyzed, and 23 of them were determined to belong to clade 2B, matching the A/South Dakota/06/2007 genomic print. Five were identified as clade 2C viruses based on their genomic prints. Seven viruses were demonstrated to match the genomic print of A/Texas/57/2009, which was previously characterized as a reassortant virus resistant to both the adamantanes and oseltamivir [28]; all seven viruses were demonstrated to contain a hybrid of BC signatures from both clades 2B and 2C. Genome sequencing, complemented with phylogenetic analyses, revealed that the majority of the dual-resistant viruses were of clade 2B that acquired the M genes from clade 2C viruses, rendering the reassortant virus resistant to both the adamantanes and oseltamivir drugs [25,26,28]. The two remaining viruses had genomic prints that did not match those from any of the three reference viruses. Of note, the BC signatures obtained from NS1 and NS2 targets in the current RT-PCR/ESI-MS assay did not allow distinction between viruses of clades 2B and 2C.

Detection of emerging or unusual influenza A viruses

More recently, using the RT-PCR/ESI-MS assay, the Naval Health Research Center (NHRC; San Diego, CA, USA), in collaboration with the CDC's Border Infectious Disease Surveillance Project, detected one of the first cases of 'unsubtypable' influenza A viruses (by rRT-PCR), which later became known as the H1N1 pandemic virus [29,30]. The virus had unique BC signatures, which were close matches to BC signatures of different genes of viruses of human, swine and avian origins. While the results obtained using this assay were not (and could not be) as definitive as full-genome sequence analyses [31], they undoubtedly indicated the presence of an atypical influenza A virus. Soon after the genome sequences of the viruses from the first cases were available, and similar strains were detected in other locations in North America [32], the unique genomic print of the prototype virus (known as A/California/04/2009) was added to the Ibis Biosciences' DB and became the reference virus for the characterization of core genes of subsequent pandemic viruses. The RT-PCR/ESI-MS assay was also independently used at the CDC during the early stages of the 2009 pandemic to confirm its ability to reliably identify the core genes of the newly emerged virus and for monitoring potential changes in the internal gene composition due to reassortment. To this end, 285 H1N1 pandemic viruses, including 209 clinical samples, were tested. The results were, overall, consistent with those obtained using the CDC rRT-PCR assay [18,102], with approximately 94% sensitivity and 97% specificity. The findings of this study suggested no significant variations in target sequences among these viruses compared with the reference virus A/California/04/2009 [18].

Differentiation between live-attenuated influenza vaccine strains from wild-type viruses

The RT-PCR/ESI-MS assay was further used to successfully differentiate between the live-attenuated influenza vaccine (LAIV) strains and the wild-type viruses [18]. RT-PCR/ESI-MS testing of a sample submitted to the CDC for routine characterization (by rRT-PCR) revealed a genomic print that was distinct from those of wild-type H1N1 pandemic, seasonal H1N1 and seasonal H3N2 viruses, as well as other viruses of animal origin. As expected for the LAIV strain of the H1N1 pandemic, the genomic print matched that of A/Ann Arbor/6/60 (H2N2), the master donor of the core genes, present in the formulation of the H1N1 pandemic LAIV. The RT-PCR/ESI-MS assay results confirmed those obtained based on rRT-PCR and gene-sequencing data [SHU B, PERS. COMM.].

Expert commentary

The RT-PCR/ESI-MS technology was first developed and used in 2005 for rapid microbial detection and identification of clinical specimens and environmental samples [9–11]. Rapidly, the method found its usage widen to include detection of biothreat agents and bacterial genotyping, as well as virus characterization (e.g., alphaviruses, coronaviruses and influenza viruses) [9–13,33,34]. In the field of influenza, a RT-PCR/ESI-MS assay was designed to target the core gene segments and not the highly variable surface antigen-coding genes, *HA* and *NA* [12,13]. Testing of the primer

sets demonstrated that they could generate PCR amplicons from a wide variety of influenza virus types/subtypes for subsequent BC signature and genomic print determination based on the core genes. The generated genomic prints are information rich and allow differentiation among the core genes of the various influenza types and subtypes [12]. The assay was subsequently used in the analysis of different genetic groups of seasonal H3N2 and H1N1 influenza viruses [12,18] and, more significantly, was used to detect one of the first two cases of the now known H1N1 pandemic virus [29,30]. It is worth noting that the initial identification of the origin of each gene of this complex reassortant virus was deficient. This inaccuracy in determining the exact sources of each gene was primarily owing to the absence of adequate swine reference sequences in the DB used for analysis. It is also essential to emphasize that the lack of systematic surveillance of swine populations for influenza added more complexity and difficulty to the identification of the closest gene ancestors of the H1N1 pandemic virus [31,35], a factor not unique to the RT-PCR/ESI-MS assay.

It is important to note that influenza A viruses consist of a segmented negative-sense RNA genome, which is prone to acquisition of point mutations and gene reassortment [36]. To date, 16 HA and nine NA subtypes of influenza A viruses have been characterized, and all have been isolated from aquatic birds [37–39]. Therefore, aquatic birds are considered the primary reservoirs for all influenza A virus subtypes. It is believed that viruses from these birds can cross species barriers and infect a large variety of animals, including humans, pigs, dogs, horses, sea mammals and birds, and result in the genesis of novel viruses [39,40].

Owing to the diversity and complexity of influenza A viruses' hosts, any attempt to determine the exact origin of the individual components of the genome of novel influenza viruses should be carried out with great caution, especially if reassortment of gene segments is involved.

The development of molecular methods that allow the rapid detection and identification of novel or unusual influenza viruses in humans is central in combating and containing future epidemics and potential pandemics. The RT-PCR/ESI-MS assay lends itself for such needs and fits a niche with the potential for significant expansion. The recent findings that reassortment between H5N1 and seasonal H3N2 viruses could result in highly virulent strains [41–43] and the isolation of a triple reassortant swine H1N1 virus that acquired the *HA* and *NA* genes from human seasonal virus [44] underline the importance of the availability of molecular tools, including the RT-PCR/ESI-MS assay, which allow rapid identification of novel strains with such complex genomes.

When compared with the more widely used rRT-PCR-based assays [2–5], which typically provide more rapid and specific results, the RT-PCR/ESI-MS assay is better suited for high-throughput settings and broad-range identification. It has the advantage of simultaneously analyzing six gene segments or more. Furthermore, the rRT-PCR-based methods would require gene sequencing in addition to phylogenetic analysis for final confirmation. Often, the goal of most rRT-PCR assays is more definitive and seeks to determine whether a specific strain (e.g., an H3N2, H1N1, H5N1 or B virus) is present in a sample. On the other hand, the RT-PCR/ESI-MS assay

aims at identifying 'what is in the sample to be analyzed', which provides an all-inclusive, although less-precise, answer. For instance, if the sample contains a virus that has been characterized previously with a unique genomic print in a reference DB, it will be identified as such. If it is a novel virus without a match in the DB, the assay will indicate that this is an atypical virus and the user could follow-up with further characterization. Such was the approach with the initial cases of the H1N1 pandemic virus [29,30]. It should be noted that the accuracy and high-throughput nature of the RT-PCR/ESI-MS assay makes it well suited for screening large numbers of viruses for genome changes, in addition to occurrences of segment reassortment. Finally, the sensitivity of the RT-PCR/ESI-MS assay, which is determined by the RT-PCR step, allows sample testing directly from clinical specimens without the need of culturing viruses, thus making it both time efficient and cost effective.

Although the RT-PCR/ESI-MS assay can be useful in influenza virus research, it is not without limitations, the most noticeable being the lack of targets for the two surface antigens, HA and NA, in the currently available Influenza Surveillance Kit. It is based on the results obtained from the core genes that the HA and NA subtypes are deduced. Even though such a correlation between the core genes and the HA and NA subtypes does exist in the vast majority of viruses circulating in humans, it is significant to note that this correlation can be compromised due to natural reassortment events or to artificial ones, such as generation of LAIV strains. Additionally, the kit does not include primers to amplify and analyze the *PB2* gene of influenza A viruses. It is worth noting that these drawbacks do not imply flaws in the technology itself but are a result of the purpose for which the assay was first designed. At that time, the goal was to demonstrate that the assay provides accurate, rapid and high-throughput detection of any influenza virus in a sample. We believe the assay could be tailored to suit more specific needs of influenza genomics, surveillance and diagnostics. Another limitation of the RT-PCR/ESI-MS assay, as currently used, is the dependency of the users on the instrument vendor for data analysis. It would be beneficial if the end users could readily access the DB used for analysis and keep it up-to-date with new reference BC signatures, genomic prints and relevant virus attributes, thereby maintaining the DB content as relevant and current.

Another shortcoming of the assays described in this study is the lack of testing for anti-influenza drug-resistance markers. However, the next-generation influenza assays in Abbott's portfolio include detection of some known markers of antiviral resistance. Nevertheless, this is not a platform limitation, since microbial resistance testing and characterization using PLEX-ID has been demonstrated for bacteria [45–47].

Noteworthy, type B influenza viruses were not discussed extensively in this article because of the limited data on the use of the RT-PCR/ESI-MS assay in analysis of these viruses. Nevertheless, it is important to emphasize that the assay is capable of detecting such viruses.

Five-year view

Since its introduction, the use of the RT-PCR/ESI-MS technology in pathogen characterization is on the increase. Although

the technology was initially intended for bacterial detection, it was quickly adapted for other applications, including influenza virus identification. In the next 5 years, the influenza RT-PCR/ESI-MS assay, in particular, is likely to gain wider usage and acceptance, especially if additional tailored kits, such as the '2009 H1N1 Pandemic Diagnostics Kit' currently being evaluated, become available. The influenza RT-PCR/ESI-MS assay could, in the future, be refined by designing multiple sets of primers to target each gene, or by including primers targeting the HA, NA and PB2 of influenza A viruses, as well as other primers to address specific questions. For example, new assays could be designed for the analysis of gene reassortment among influenza viruses in pigs, or for the investigation of reassortment between the H1N1 pandemic and highly pathogenic H5N1 viruses and determination of which gene segments are involved in a particular reassortment event.

In the near future, the RT-PCR-ESI-MS assay has the potential of becoming a valuable addition to the array of tools used in influenza virus surveillance and diagnostics. Recently, the kit currently in use was customized to develop a diagnostic assay for the H1N1 pandemic viruses. Validation, approval and

expansion of this diagnostic kit to include other subtypes could be of significant clinical relevance, especially since the assay can be performed directly on clinical specimens. Availability of such capabilities would aid in improving the management of patients.

FIGURE 5 illustrates the design of this new assay. All eight segments were targeted including the PB2 for influenza A viruses, and HA and NA, which are targeted to the H1N1 pandemic. The PB2 and HA primers replaced the influenza A virus NS2 and influenza B virus NP targets, respectively, while the NA primers were duplexed with those targeting the influenza B virus PB2 gene. Details of the primers in the new assay are presented in TABLE 2. Such improvements and refinements in the assay can be challenging; however, they could be valuable in providing a rapid 'snap shot' of the entire genome with a high-throughput method and, subsequently, a more in-depth virus characterization using the assay's built-in neural network capability.

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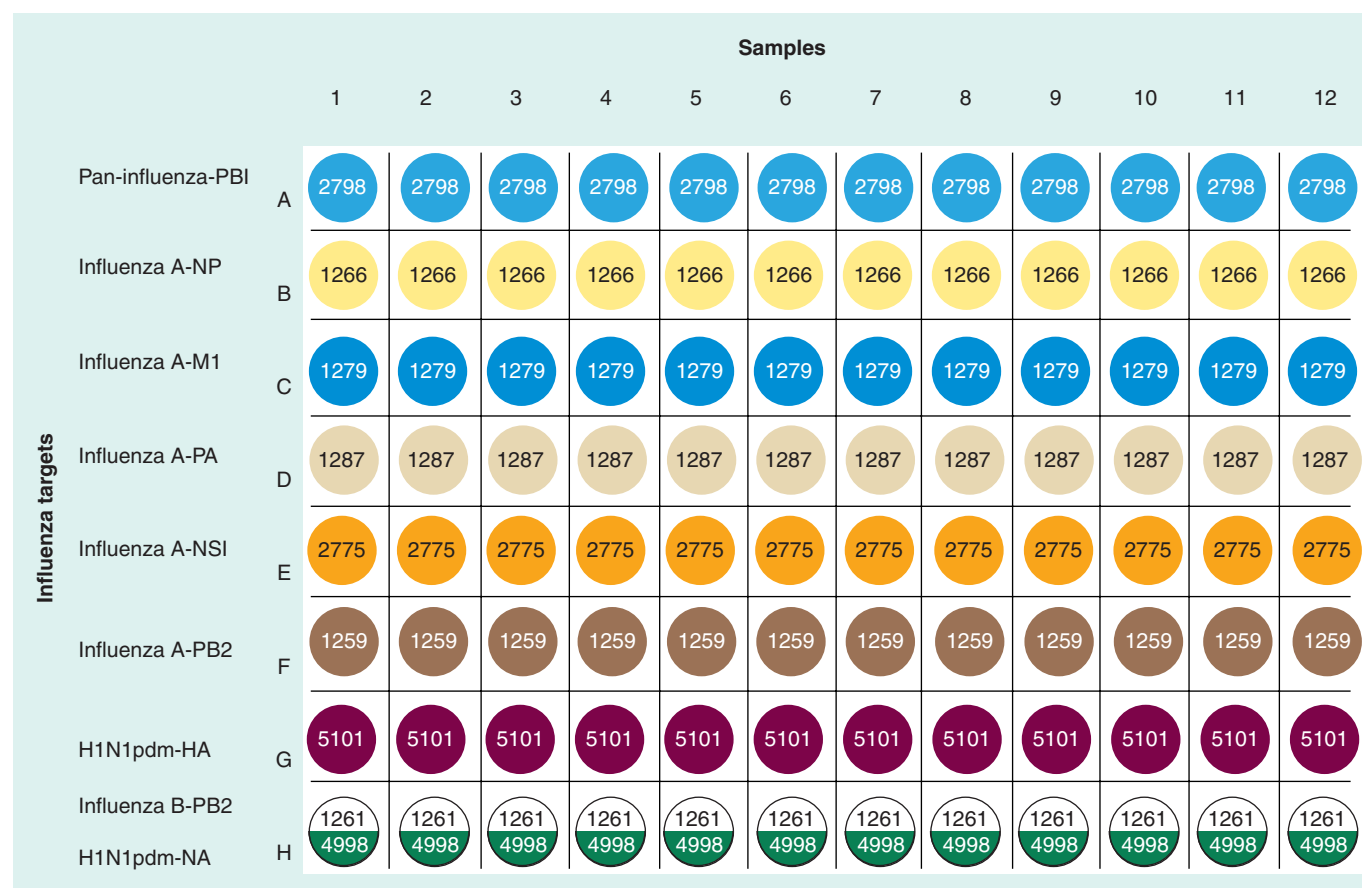


Figure 5. Customized reverse-transcription-PCR/electrospray ionization mass spectrometry assay for the diagnosis of the H1N1 pandemic virus. New influenza kit under evaluation features replacement of the influenza A NS2 and influenza B NP primers used in the old version with influenza A PB2 (1259) and the H1N1 pandemic virus HA (5101), respectively. Additionally, H1N1 pandemic NA primers (4998) are duplexed with the influenza B PB2 gene primer set in the last row (H1–H12) of the plate. Details on the new primers are shown in TABLE 2. The remaining primers are the same as those described in FIGURE 1 and TABLE 1. H: Hemagglutinin; N: Neuraminidase.

Table 2. H1N1 pandemic virus reverse-transcription PCR/electrospray ionization mass spectrometry diagnostics assay primer sets.

Primer ID	Gene segment targeted	Influenza	Primer sequence	Amplicon length (bp)
2798	PB1	A, B, C	F: TGCCTGGAATGATGATGGGCATGTT R: TCATCAGAGGATTGGAGTCCATCCC	128
1266	NP	A	F: TACATCCAGATGTGCACTGAACTCAAACCTCA R: TCGTCAAATGCAGAGAGCACCATTCTCTCTA	111
1279	M1	A	F: TCTTGCCAGTTGTATGGGCCTCATATAC R: TGGGAGTCAGCAATCTGCTCACA	115
1287	PA	A	F: TGGGATTCCTTTCGTCAGTCCGA R: TGGAGAAGTTCGGTGGGAGACTTTGGT	122
2775	NS1	A	F: TCCAGGACATACTGATGAGGATGTCAAAAATGCA R: TGCTTCCCAAGCGAATCTCTGTA	129
1259	PB2	A	F: TACCACTGTGGACCATATGGCCATAAT R: TCGGATATTCATTGCCATCATCCACTTCAT	105
5101	HA	A/H1N1 pandemic	F: TGGACAGGGATGGTAGATGGATGGTACGG R: TCTCGTCAATGGCATTCTGTGTGCT	103
4998	NA [†]	A/H1N1 pandemic	F: TCAGTCGAATTGGATGCCCTAATTA R: TATGAACTCAGCTGATGTTGCTCCTGC	73
1261	PB2 [†]	B	F: TCCCATTGTACTGGCATACTGCTTGA R: TATGAACTCAGCTGATGTTGCTCCTGC	101

Primer pairs targeting PB1, NP, M1, PA and NS1 are the same as the ones described in TABLE 1. Primer set 1259 is added to target all influenza A viruses. Primer pairs targeting hemagglutinin and neuraminidase are designed to identify the H1N1 pandemic viruses.

[†]These two primer sets were duplexed (included) in the same well.

F: Forward; ID: Identification; R: Reverse.

Financial & competing interests disclosure

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Key issues

- The influenza reverse-transcription (RT)-PCR/electrospray ionization (ESI) mass spectrometry (MS) assay kit provides a rapid and broad-range influenza virus 'core' gene-characterization tool.
- The assay is based on ESI-MS and analysis of the base composition of RT-PCR amplicons generated using primers targeted to highly conserved regions flanking highly variable sequences in the influenza core's genes.
- The assay is high throughput and allows analysis of approximately 300 samples in 24 h (from specimen receipt to reporting results).
- One of the first two human cases of a reassortant virus, now known as the H1N1 pandemic virus, was identified using this assay.
- The RT-PCR/ESI-MS assay, in its current format, is designed for the broad-range detection of influenza A and B viruses based on the core genes, but can be tailored for the specific needs of surveillance, as well as for diagnostic purposes.
- The premise of this assay is that it not only permits rapid characterization of previously known viruses, but it also detects unusual and emerging influenza viruses, based on their core genes' signatures.
- The level of accuracy in virus characterization depends upon the reference base-composition signatures and genomic prints present in the database at the time of analysis.

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