Simplified Large-Scale Sanger Genome Sequencing for Influenza A/H3N2 Virus

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Abstract

Background: The advent of next-generation sequencing technologies and the resultant lower costs of sequencing have enabled production of massive amounts of data, including the generation of full genome sequences of pathogens. However, the small genome size of the influenza virus arguably justifies the use of the more conventional Sanger sequencing technology which is still currently more readily available in most diagnostic laboratories.

Results: We present a simplified Sanger-based genome sequencing method for sequencing the influenza A/H3N2 virus in a large-scale format. The entire genome sequencing was completed with 19 reverse transcription-polymerase chain reactions (RT-PCRs) and 39 sequencing reactions. This method was tested on 15 native clinical samples and 15 culture isolates, respectively, collected between 2009 and 2011. The 15 native clinical samples registered quantification cycle values ranging from 21.0 to 30.56, which were equivalent to $2.4 \times 10^3 - 1.4 \times 10^4$ viral copies/µL of RNA extract. All the PCR-amplified products were sequenced directly without PCR product purification. Notably, high quality sequencing data up to 700 bp were generated for all the samples tested. The completed sequence covered 408,810 nucleotides in total, with 13,627 nucleotides per genome, attaining 100% coding completeness. Of all the bases produced, an average of 93.46% were QV30 bases (one miscall every 1000 bases) or higher, and an average of 89.49% were Phred quality value 40 (QV40) bases (representing an accuracy of circa one miscall for every 10,000 bases) or higher.

Conclusions: This sequencing protocol has been shown to be cost-effective and less labor-intensive in obtaining full influenza genomes. The constant high quality of sequences generated imparts confidence in extending the application of this non-purified amplicon sequencing approach to other gene sequencing assays, with appropriate use of suitably designed primers.

Introduction

In recent years, advances in sequencing techniques have enabled an increasing number of research studies based on the genome-wide sequences of the influenza viruses [1–6], rather than relying solely on an individual gene that may preclude more comprehensive gene signatures [7,8]. Since the large number of influenza genome sequences deposited by Ghedin et al. [4] and the initiation of the Influenza Genome Sequencing Project in 2005 [9], the deposition of complete human influenza A virus genomes by other groups has increased exponentially.

The genome of the influenza A virus (family Orthomyxoviridae) consists of eight segmented, negative-stranded RNAs, ranging from 890 to 2,341 nucleotides (nt), constituting 13,627 nt per genome. The eight RNA segments encode for (in the order of the segment numbers one to eight): viral RNA polymerase basic 2 (PB2, 2341 nt), polymerase basic 1 (PB1, 2341 nt), polymerase acidic (PA, 2233 nt), hemagglutinin (HA, 1762 nt), nucleoprotein (NP, 1567 nt), neuraminidase (NA, 1466 nt), matrix (M1, 1027 nt), and nonstructural (NS1, 890 nt) protein. Apart from these proteins, alternatively spliced mRNAs of the seventh segment (M1) and the eighth segment (NS1) allow translation of two additional proteins, namely, the ion channel matrix protein (M2) and nuclear export/nonstructural protein (NEP/NS2), respectively. Also, PB1-F2 proteins are alternatively translated from PB1 gene segments of some influenza A viruses [10].

The introduction of next-generation sequencing (NGS), which delivers high throughput readings [11] compared to the traditional Sanger dideoxy chain-termination method [12], has provided a remarkable cost reduction for microbial genome sequencing. However, a higher error rate due to homopolymeric miscalling and other systematic base-calling biases have been observed in NGS techniques, compared with the Sanger methods [13–16]. The average error rate of the former is considerably higher, with a value of $10^{-2} - 10^{-3}$ versus that of the latter at $10^{-4} - 10^{-5}$ [13,14]. A recent report on 12 influenza genomes comparing 2 NGS
platforms from 454 Life Sciences and Illumina revealed error rates up to $10^{-3}$ and $10^{-5}$ at the homopolymeric region, respectively [17]. Besides, the cost of the initial NGS capital equipment outlay, together with the additional bioinformatics manpower support for the storage and analysis of the huge amount of data generated through the NGS system [18] may not be cost-effective for many smaller research laboratories for the sequencing of influenza viruses which have a relatively small genome size (~14 kb).

The Sanger technique is regarded to be low throughput and more tedious, due to the requirement of multiple purification or plasmid cloning steps [4,8,19–23]. Here, we describe a whole genome sequencing method for seasonal influenza A/H3N2, with modifications of the normal sequencing protocol that reduces the number of processing steps, but still constantly produces a high quality sequence read of up to 700 bp. This protocol, when applied systematically, should hasten the routine genome sequencing work for local influenza surveillance studies. It was also demonstrated that this protocol is highly applicable for both clinical samples and Madin-Darby canine kidney- (MDCK-) cultured samples.

### Results

#### Clinical Specimens and Culture Isolates

A total of 30 archived influenza A/H3N2 clinical samples collected from different patients between 2 May 2009-1 Aug 2011 were selected randomly for this study. All samples were received for diagnostic testing at the National University Hospital (NUH) in Singapore and were confirmed positive using two clinically validated, in-house, real-time influenza A/B screening [24] and subtyping assays [25,26]. The samples included nasal/nasopharyngeal or throat swabs collected in universal transport medium, endotracheal tube aspirates, or sputum samples. Fifteen of the 30 were sequenced from cultured isolates of the original clinical sample using a MDCK.2 (ATCC; CRL-2936) cell line; the other 15 sequences were obtained directly from the clinical samples with no preliminary culture step.

#### Primer Design

To ensure the utility of the assay for the sequencing of older as well as future circulating strains, two reference gene sequences were randomly chosen per month from depositions from different countries and dates of collection (2007 to 2011) available at the NCBI Influenza Virus Resource. Primer target regions for RT-PCRs for the different gene segments were selected from the conserved regions of the respective aligned gene sequences. Large gene segments (1 to 3) were amplified as three fragments. Small segments (4 to 8) were amplified as two fragments. To achieve tolerance for accurate sequence assembly, the PCR products for each of these segments overlapped with preceding and follow-up segments for at least 39 bp. The 5’ and 3’ ends of each segment were amplified using modified published forward (MBTuni-12) and reverse (MBTuni-13) primers [21,27]. Sequencing primers were designed within the internal regions of the PCR products. All the sequencing and RT-PCR primers are listed in Tables 1 and 2, respectively.

#### PCR Sensitivity

The 15 RNA samples extracted directly from the clinical samples were of quantification cycle values ranging from 21.0 to 30.56 (equivalent to 2.4×10^{-1}–1.4×10^{6} viral RNA copies/μL of RNA extract) [24]. All of the gene segments from both the clinical and MDCK-cultured samples collected from 2009-2011 were successfully amplified and appeared as specific and discernible bands on the agarose gel. It was noticed that some gene amplifications additionally produced minor non-specific bands in clinical samples with low viral titers.

### Sequencing

All the eight segments from the respective 15 clinical and MDCK-cultured samples were successfully sequenced with high Phred quality value (QV) [28], and sequencing length up to 700 bp (Table 1). Length of read (LOR) for all sequence contigs had base calls of QV20 (representing an accuracy of circa one miscall for every 100 bases) and above for at least 20 continuous bases, which was in accordance to the analyzer machine’s default setting. Sequences with a mixture of nucleotides that contained only a single coverage depth was confirmed with reverse sequencing using PCR primers from the purified amplicon method briefly described in Figure 1. In total, the completed sequences obtained from the 15 cultured isolates and directly from the 15 clinical samples covered 408,810 nucleotides, with 13,627 nucleotides per genome, attaining 100% coding completeness. The entire sequencing protocol produced an average of 1.57 sequencing reads covering each nucleotide. Of all the bases in the assembly, an average of 89.49% were QV40 bases (representing an accuracy of circa one miscall for every 10,000 bases) or higher, and an average of 93.46% were QV30 bases (one miscall every 1000 bases) or higher (Table 1). All the sequences were successfully assembled into their respective segments. The use of the non-purified amplicon method resulted in a very high-quality genome assembly, including samples that had Ct values up to 30. The total sequencing raw data obtained per genome was less than 5 megabytes of data storage. The sequence analyses and assembly for each genome was completed within 15–30 minutes.

The sequencing chromatograms generated were uploaded into Trace Archive [trace identifier number: 2333373621–2333374798] to allow visual inspection of the traces and quality scores underlying every nucleotide in each of the thirty genomes [29,30]. All assembled sequences obtained in this study were uploaded onto NCBI GenBank [accession number: JX437693-JX437932].

#### Further Testing of Assay Protocol on other Clinical Samples

The genome sequencing and assembling protocols were further tested on 125 additional H3N2 primary clinical samples with Ct values of 30.56 and below. All the 125 samples were collected in NUH as diagnostic samples from 1 May 2009–15 Dec 2012. Of the 125 additional primary clinical samples, 118 were sequenced and assembled completely. In total, 134 out of 140 (96%) primary clinical samples were successfully sequenced in this study with similar Phred quality.

There were seven samples that could not be sequenced completely. More specifically: full PB2, PB1, PA, HA, NP, and NS sequences were not obtainable from 2, 3, 3, 2, 1, 2 of these seven samples, respectively. Of these 13 failures, nine were from two samples with Ct values of 28.72 and 29.04, respectively. The PB1 and PA genes encountered the highest failure rate relative to the others.

### Discussion

Traditionally, Sanger sequencing is performed on purified PCR amplicons to prevent background noise generated during sequencing analyses. Here, it was found possible to employ a non-purified amplicon approach for direct sequencing, which minimized processing time and effort for large-scale viral genome sequencing that produced consistently high quality sequencing...
| Segment/fragment | Primers   | Primer sequence (5’-3’) | Nucleotide position (5’-3’) | Reference | Average percentage of bases ≥QV40 (S.D.) | Average percentage of bases ≥QV30 (S.D.) | Mean LOR in bases (S.D.) |
|------------------|-----------|-------------------------|-----------------------------|-----------|------------------------------------------|------------------------------------------|------------------------|
| 1(PB2)/A         | PB2_230F25| CGGAGAAGAATGACACCGAACCAC | 230–254                     | GU907121  | 91.62 (5.62)                             | 94.46 (4.80)                             | 556 (23)               |
|                  | PB2_629R26| TTCTCTAAGATGTGGAGACCTCA  | 654–629                     | 89.87 (7.45) | 94.45 (5.05)                             |                                          | 593 (24)               |
| 1(PB2)/B         | PB2_960F21| CAARGCTGCAATGGATGAAAG    | 960–980                     | 89.93 (5.82) | 94.33 (4.69)                             |                                          | 618 (23)               |
|                  | PB2_1432R24| TCTCTGACATCTGGTCTGG     | 1455–1432                   | 90.00 (6.36) | 94.31 (4.83)                             |                                          | 597 (24)               |
| 1(PB2)/C         | PB2_1796F25| GCCAAATAGGGTTGTTCTGAAC   | 1796–1820                   | 92.69 (3.74) | 94.58 (3.37)                             |                                          | 498 (17)               |
|                  | PB2_2118R25| TCCRTAYCTTCTTGCTCTCT    | 2142–2118                   | 89.27 (4.83) | 93.79 (4.11)                             |                                          | 580 (21)               |
| 2(PB1)/A         | PB1_232F25| GATGGACCACTACCTGAGGAATG  | 232–256                     | AB441948  | 91.70 (3.96)                             | 94.56 (3.93)                             | 540 (21)               |
|                  | PB1_590R23| GGTATGGCTGCTCCTCTCTGCTTC| 612–590                     | 89.39 (5.70) | 93.43 (4.77)                             |                                          | 552 (24)               |
| 2(PB1)/B         | PB1_1007F26| ATCAATCGGAGTTGGTTCAAGATCTC| 1007–1032                  | 86.18 (5.45) | 92.83 (4.38)                             |                                          | 681 (23)               |
|                  | PB1_1369R26| TCTATGCTGTCAGTCTATGAG    | 1394–1369                   | 90.25 (5.11) | 93.72 (4.52)                             |                                          | 582 (26)               |
| 2(PB1)/C         | PB1_1700F25| ATAGRTGCAATGGAGGAGGAGACA| 1700–1724                   | 91.20 (3.87) | 94.93 (3.49)                             |                                          | 579 (22)               |
|                  | PB1_2126R25| ATGCTGTCTCTATGACTACTAG   | 2150–2126                   | 89.21 (6.23) | 94.24 (5.56)                             |                                          | 627 (31)               |
| 3(PA)/A          | PA_210F24 | GGTGTAACCTTGCRACTCACAATGC| 210–233                     | GU907117  | 90.40 (7.02)                             | 93.96 (4.66)                             | 520 (21)               |
|                  | PA_601R23 | GGTCTCTCGCTCTTCTCCTGCTG| 623–601                     | 89.82 (5.04) | 92.96 (4.66)                             |                                          | 559 (25)               |
| 3(PA)/B          | PA_862F23 | TCAARTCTCTCCTCGATAGTATGC| 862–884                     | 90.78 (3.85) | 94.85 (2.96)                             |                                          | 641 (18)               |
|                  | PA_1225R24| CTGATGCCTGAGTTGGGCAAGATTC| 1248–1225                  | 91.55 (8.56) | 94.21 (7.83)                             |                                          | 493 (38)               |
| 3(PA)/C          | PA_1608F20| TGACCCGAGAATCCAGACCAC   | 1608–1627                   | 92.89 (3.16) | 95.71 (2.73)                             |                                          | 572 (13)               |
|                  | PA_1975R24| AAACCTTCCATTGTGCTGATGC  | 1978–1975                   | 90.37 (6.22) | 93.16 (8.56)                             |                                          | 544 (68)               |
| 4(HA)/A          | HA_286F24 | TATTTGAGGACCTCCATTGTGATG| 286–309                     | GU907114  | 88.85 (6.64)                             | 94.39 (3.70)                             | 689 (16)               |
|                  | HA_517R27 | GGTTGACCATTCAATTCAATTAAAGA| 543–517                    | 89.77 (6.92) | 93.20 (6.23)                             |                                          | 491 (22)               |
| 4(HA)/B          | HA_1387F26| TTGCATCTACCTGACATGAAATGAC| 1387–1412                  | 88.61 (5.25) | 91.77 (4.79)                             |                                          | 324 (17)               |
|                  | HA_1393R27| ACAGTGTGTCATTCTCAGTCTGAGTTA| 1419–1393                | 85.39 (14.55) | 91.35 (10.33)                             |                                          | 474 (18)               |
|                  | HA_1632R25| GCAAAAAACATGATGTCACGCAAGAGA| 1656–1632                  | 75.69 (11.79) | 86.43 (8.36)                             |                                          | 707 (26)               |
| 5(NP)/A          | NP_1662F5 | ATCCAAATGGGTCAGTAACTAAC | 166–190                     | GU907120  | 88.28 (8.19)                             | 94.00 (5.24)                             | 653 (25)               |
|                  | NP_664R20 | GGYGCTYTGCACTCCCTCCCTCCA| 681–664                     | 91.71 (5.63) | 95.21 (4.06)                             |                                          | 624 (23)               |
| 5(NP)/B          | NP_998F25 | CTACGCAGGAGCAGCAGCAAGAGA| 998–1022                    | 90.46 (4.60) | 93.66 (4.17)                             |                                          | 507 (20)               |
|                  | NP_1522R23| GCCATATTCTTCATGCTCGCTCACA| 1344–1322                  | 88.65 (7.16) | 93.33 (5.47)                             |                                          | 520 (27)               |
| 6(NA)/A          | NA_350F20 | GGYGGRACATCTGGTGGTGCT   | 350–369                     | GU907119  | 90.32 (4.46)                             | 93.53 (3.39)                             | 480 (17)               |
|                  | NA_529R23 | ATCACTATGACCCAGTGCTGCTGC| 551–529                     | 88.24 (10.79) | 92.38 (8.81)                             |                                          | 494 (24)               |
| 6(NA)/B          | NA_699R25 | CTAATGATACACGCTATGCTGACT| 723–699                     | 87.70 (6.05) | 93.66 (3.37)                             |                                          | 667 (19)               |
|                  | NA_1090F24| AAAAGCTGTCGGATGGAGGAGGC| 1090–1113                   | 88.12 (7.04) | 91.74 (6.11)                             |                                          | 322 (17)               |
|                  | NA_1331R24| CACCAAACTTGGTGACCTGCTCA| 1354–1331                   | 90.43 (5.87) | 94.36 (3.94)                             |                                          | 584 (20)               |
| 7(MP)/A          | MP_78F18  | GCCGCGCTCAAGGCGCAGA      | 78–95                       | GU907115  | 89.32 (5.56)                             | 92.81 (1.93)                             | 457 (17)               |
|                  | MP_551R23 | CTGGGCAARACATCTCCTGCTCTC| 573–551                     | 90.42 (4.21) | 94.10 (1.38)                             |                                          | 520 (17)               |
The performance of each sequencing primer is described in Table 1, as seen by the average percentage of bases generated from the 30 complete genomes with QV more than 30 and 40, respectively. The QV values were generated using the proprietary sequencing analysis software (version 5.2) of the ABI 3130 genetic analyzer (Applied Biosystems). Length of Read (LOR) is defined as the length of sequence with QV20 and above for at least 20 continuous bases.

The entire genomic sequencing for the influenza A/H3N2 virus can be completed with a data storage size of approximately 5GB.
megabytes per genome, permitting convenient data handling by biologists or non-bioinformatics expertise for large-scale sequencing for local surveillance purposes. The sequencing cost per genome of the entire protocol from RNA extraction to sequence analysis was calculated to be less than SGD 350 (USD 290), compared to the conventional purified-amplicon method at around SGD 410 (USD 340) and plasmid cloning approach at roughly SGD 1360 (USD 1120). The high quality data obtained from multiple sequencing reactions targeting different genes (Table 1) suggested the applicability of this technique for other viral (i.e., small genome) gene sequencing work.

Influenza surveillance will continue on a worldwide basis for the foreseeable future, and molecular surveillance for influenza using partial or full-genome sequencing is now becoming routine in many diagnostic laboratories—especially in those which are not set up to perform the traditional serological surveillance for influenza.

| Segment/fragment | Primers | Primer sequence (5’-3’) | Nucleotide position (5’-3’) | Reference gene | Second T<sub>s</sub> (°C) |
|------------------|---------|-------------------------|---------------------------|---------------|---------------------|
| 1(PB2)/A         | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907121      | 59                  |
|                  | PB2_841R24 | AGATGCCAGCTGCTGCTGAC   | 864–841                   |               |                     |
| 1(PB2)/B         | PB2_778F24 | AGAATGCGATGTGACCAAAAAGC | 778–801                  |               |                     |
|                  | PB2_1631R24 | CAGGACCTGAATCTCCCACTCA | 1654–1631                |               |                     |
| 1(PB2)/C         | PB2_1501F22 | GAGAGGGTGTTGAGATTGACTG | 1501–1522                |               | 59                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 2(PB1)/A         | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | AB441948      | 60                  |
|                  | PB1_820R21 | GGAAGCTGACTGTTGAGAGCG  | 840–820                   |               |                     |
| 2(PB1)/B         | PB1_733F23 | AARAGAAGGCATGGCAAAAGCAG | 733–755                  |               |                     |
|                  | PB1_1765R23 | CCGTTGACTGTTATGATGCTC | 1787–1765                |               |                     |
| 2(PB1)/C         | PB1_1447F25 | ATCAACATGAGAACAAAGTCTT | 1447–1471                |               | 58                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 3(PA)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907117      | 61                  |
|                  | PA_778R25 | AAGTTCATTTGGCAGTCCTCTT | 802–778                  |               |                     |
| 3(PA)/B          | PA_683F21 | CACCCGATTTCCTGCTGCTG | 683–703                   |               | 58                  |
|                  | PA_1558R24 | ATTTACACCTGCTGATCTCCTT | 1581–1558                |               |                     |
| 3(PA)/C          | PA_1416F23 | CTTAACACTGCTGCTCTCAAGT | 1416–1438                |               | 59                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 4(HA)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907114      | 61                  |
|                  | HA_1013R23 | YCCTGTTGGCAGTCCTCTTCG  | 1034–1013                |               |                     |
| 4(HA)/B          | HA_873F25 | TCAATAATGAGATCAGATCAAG | 873–897                  |               |                     |
| MBTuni-13 |                  |                          |                         |               |                     |
| 5(NP)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907120      | 61                  |
|                  | NP_868R18 | CGCACAAGGGCTAGAGCGCA  | 885–868                  |               |                     |
| 5(NP)/B          | NP_753F23 | AGAATGCGATGCTAGATGAG  | 753–775                  |               | 60                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 6(NA)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907119      | 59                  |
|                  | NA_862R23 | ATCTGACACAGGRTATCCGGGA | 884–862                  |               |                     |
| 6(NA)/B          | NA_699F25 | AATCCGATGCTGCTGCTGCTA | 699–723                  |               | 58                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 7(MP)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907115      | 61                  |
|                  | MP_582R23 | AGCATTGTTGATCCTGCTAGCTT | 604–582                  |               |                     |
| 7(MP)/B          | MP_429F21 | TGGGCGGTAGACCTGAGAG  | 429–449                  |               | 59                  |
| MBTuni-13 |                  |                          |                         |               |                     |
| 8(NS)/A          | MBTuni-12 | ACGCGTGATCGCAAAAGCAGG | 1–12                      | GU907116      | 60                  |
|                  | NS_464R22 | CTCCTCAGGTAAAGCCTTCTT | 485–464                  |               |                     |
| 8(NS)/B          | NS382F21 | TGGACCAGGCAATCAGGAGA  | 382–402                  |               | 60                  |
| MBTuni-13 |                  |                          |                         |               |                     |

The T<sub>s</sub> for all the PCR primers ranged between 58 and 61°C. MBTuni-12 and MBTuni-13 primers targeting the 5’ and 3’ ends of each segment were adopted from published methods [21,27], with nucleotides (in bold) representing the modifications made. Nucleotide R (bold) in the primer sequence indicates a degenerate nucleotide that represents A or G.

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Influenza surveillance will continue on a worldwide basis for the foreseeable future, and molecular surveillance for influenza using partial or full-genome sequencing is now becoming routine in many diagnostic laboratories—especially in those which are not set up to perform the traditional serological surveillance for influenza.
Among the different seasonal human influenza viruses, influenza A/H3N2 has circulated in the human population since its emergence during the 1968 'Hong Kong' pandemic, and has persisted successfully, despite the emergence of the 2009 A/H1N1pdm virus and its subsequent almost virtual replacement of the previously circulating seasonal influenza A/H1N1 [35,36]. Ongoing antigenic changes in circulating seasonal A/H3N2 viruses continue to trigger new recommendations for seasonal influenza vaccine composition, to optimize vaccine-induced immunity in both the community and healthcare worker populations [37–39]. Thus, ever more efficient and economical methods are required to keep down the costs of molecular surveillance, allowing more laboratories to perform such sequencing routinely, thereby enhancing the quality, temporal and geographical resolution of the local influenza surveillance data available, to keep vaccine manufacturers and public health teams informed [40]. Towards this goal, the simplified sequencing protocol described here has been shown to be effective in obtaining full influenza A/H3N2 genomes at a reasonable price with equipment already available in many diagnostic and research laboratories, suggesting potential use of a similar strategy for studying human influenza A/H1N1pdm viruses.

### Methods

#### Ethics Statement

All research studies involving the use of these clinical samples were reviewed and approved by the local institutional ethics review board (National Healthcare Group: B/09/360 and E/09/341).
Viral RNA Extraction

Viral RNAs were extracted from 200 μL of clinical or cultured samples with either the Qiagen EZ1 Virus mini kit v2.0 or the QIAasympmetry Virus/Bacteria mini kit, using their respective proprietary Bio Robot EZ1 and QIAasympmetry automated platforms (Qiagen, Valencia, CA), according to the manufacturer’s instructions. All extracted RNAs were eluted into a final volume of 60 μL of elution buffer.

Reverse Transcription Polymerase Chain Reaction

RT-PCRs were performed with a Superscript III one-step RT-PCR system with Platinum Taq high-fidelity polymerase (Invitrogen, Carlsbad, CA). Nineteen RT-PCRs were set up for whole genome amplification. All RT-PCRs were prepared manually in 10 μL of reaction volume, consisting of 5 μL of 2× Reaction Mix, equimolar amounts of forward and reverse primers (0.3 μmol/L each), 0.25 μL of enzyme mix, and 2.5 μL of extracted RNA sample. The remaining volume was topped up with RNAse-free water. All RT-PCRs were performed using either the ABI 9700 thermal cycler (Applied Biosystems, CA, USA) or the Biometra T3000 thermocycler (Biometra GmbH, Goettingen, Germany). The cycling conditions were 30 min at 42°C (RT); 2.5 min at 95°C (inactivation of RT enzyme and activation of Taq enzyme); 5 cycles of 30 s at 95°C (denaturation), 30 s at 47°C (annealing), and 1.25 min at 68°C (extension); 45 cycles of 30 s at 95°C, 30 s at the respective second annealing temperature (Ts), and 1.25 min at 68°C followed by a hold for 10 min at 68°C (final extension). The second Ts for each RT-PCR is summarized in Table 2.

Sequencing

Sequencing reactions were performed directly on non-purified amplicons, using BigDye Terminator v3.1 chemistry (Applied Biosystems). The 10 μL sequencing reaction is composed of 1.5 μL of 5× Buffer, 0.5 μmol/L of respective sequencing primer (Table 1), 1 μL of BigDye enzyme mix, and 1.25 μL of template amplicons. One microliter of 4% DMSO was added into the sequencing reaction together with primer NS373R23 [29]. Large-scale sequencing reactions were carried out on a 96-well plate and purified directly using the BigDyeXTerminator purification kit (Applied Biosystems). Individual sequencing reactions were performed in PCR tubes and purified using the DyeEx 2.0 spin kit (Qiagen). Purified sequencing products were analyzed on the ABI 3130xl genetic analyzer (Applied Biosystems) using the BDx_stdSeq50_POP7_1 run module. Sequencing peak heights were adjusted with the sample injection time ranging from 3–5 seconds.

Contig Assembly

All sequences were assembled and verified using the ATB software, version 1.0.2.41 (Connexio Genomics, Perth, Australia), using the reference sequence influenza A/Nanjing/1/2009(H3N2) for all segments (GenBank accession: GU907114-GU907117 and GU907119-GU907121), except for the PB1 segment which used influenza A/Sendai/H/F193/2007(H3N2) (GenBank accession: AB441948) as the reference sequence. The primer sequences were subtracted from the data during contig assembly. The multiple A’s observed at the 3’ end of the NA, NP, and PA genes were checked carefully by visualization of the sequencing chromatograms.

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Author Contributions

Conceived and designed the experiments: HKL JWT ESK. Performed the experiments: HKL DHK. Analyzed the data: HKL DHK. Contributed reagents/materials/analysis tools: ESK. Wrote the paper: HKL JWT DHK ESK.

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