Automation of Reverse Transcription of Avian Influenza Viral RNA and Detection of H5N1 with VERSA Mini PCR Setup Workstation

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I. Abstract

In recent years, influenza virus H5N1 is being closely monitored by public health authorities for it's potential as a major health pandemic. For the screening and detecting this virus worldwide, automation is becoming an important tool. At Aurora Biomed, automated sample preparation for the detection of viral RNA from influenza A/H5N1 was developed using a commercially available kit. Liquid handling for various components of the kit was carried out using the VERSA Mini PCR Setup Workstation. The detection of amplified products of about 191, 192, and 107bp indicate that the VERSA workstation can be used to automate the kit. In addition, these results suggest that the VERSA workstation is suitable for the precautionary requirements of the kit : RNA sensitivity to degradation, PCR process sensitive to volume changes, and contamination issues.

II. Introduction

Automation is slated to play an important role in the detection of avian influenza virus- the virus found chiefly in birds. However, natural infections with influenza A viruses have been reported in a variety of animal species including humans, pigs and birds[1]. In the ongoing effort to prevent wide-spread disease, robotic workstations with higher throughput can be used to carry out large scale detection of this virus in avian or human samples.

The H5N1 viral infection can be detected using ELISA, or PCR (end point or quantitative)[2]. The detection process for end-point and quantitative PCR involves the isolation of viral genome (RNA), conversion of RNA into cDNA which is followed by amplification of the specific target(s) from the DNA. The primary purpose of automation is, therefore, to minimize the time involved with sample preparation and reduce the cumbersome nature of the processes involved, while increasing the quality of the data. Aurora Biomed has launched the VERSA Mini PCR Setup Workstation, a cost effective tool to increase the throughput and reproducibility of the H5N1 detection processes.

III. Objectives

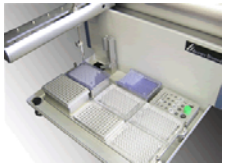
- **To check for thermostability and sensitivity to degradation of the RNA during automation**
- **To automate reverse transcription of viral RNA**
- **To check automated distribution of three, kit-based master mixes**
- **To allow amplification of determinants from different genes of viral RNA**
- **To develop a method for handling sensitive nucleic acids, like RNA**
- **To check for cross contamination or false positive/negative amplicons (if any)**

IV. Materials & Methods

The validation of VERSA Mini PCR Setup Workstation was conducted using the H5N1 detection kit supplied by Mediagnost (Reutlingen, Germany)[3], as follows:

1. Tips (1000 and 20µL) were placed at deck position 8 and 1, respectively. Tip changer sequences were performed for each reagent and DNA sample.

VERSA Mini PCR Setup Workstation



2. The vials of the lyophilized reaction mixes Rx-Mix A identification of A-type of the influenza virus), Rx-Mix H5 (for identification of hemagglutinin gene HA 5), and Rx-Mix N1 (for identification of neurminidase gene NA 1) provided in the kit were placed in the appropriate slots on the cooling block at 4°C.
3. Reverse transcriptase, *taq* DNA polymerase, sterile nuclease free water, viral RNA, and negative controls were incubated in appropriate slots of the cooling block.
4. The viral RNA sample and the negative controls were placed at deck position 5.
5. A 96-well PCR plate was placed at deck position 5.
6. The protocol sequences were carried with VERSA Mini PCR Setup Workstation.
7. The Rx-Mixes were reconstituted from lyophilized reaction mixes by adding 470 µL of nuclease free water to each of the Rx-Mix vials with the workstation and mixed with pipetting action of the workstation.
8. Aliquote of 100 µL of each of the reaction mix was transferred into vials placed on the cooling block for preparing working master mixes.
9. To each of the above-said vials containing aliquoted Rx Mix, reverse transcriptase (2 µL), and *taq*-polymerase (0.8 µL) were added.
10. To set up 25 µL reactions, 22.5 µL from Rx-Mix A was distributed by the workstation into wells of the PCR plate designated in the automation sequence. Similarly, the same volume from Rx-Mix H5, and Rx-Mix N1 was distributed.
11. Viral RNA (2.5 µL) was then added to the appropriate wells of the above-said PCR plate containing the reaction mixes and enzymes. Similarly, the same volume of the negative controls was also added to the respective wells of the PCR plate. Pipetting action was used for mixing.
12. The entire automated operation was carried inside the HEPA Hood to avoid air-borne contamination.
13. Reverse transcription and amplification were carried on MyCycler (BioRad Labs) as shown in Table 1.
14. The amplified product was detected with ethidium bromide on agarose gel (3%).

Table 1: **Conditions for reveres transcription and cDNA amplifications**

Step	Sub-step	Temperature °C	Duration Min
Reverse Transcription		42	60
Initial denaturation		94	3
Cycling 35X			
	Denaturation	94	1
	Annealing	55	1
	Extension	72	1
Final elongation		72	5

V. Results

The results from the robotic automation of reverse transcription of avian flu RNA and amplification of cDNA along with detection of H5N1 flu are presented in the form of questions and answers supported by the data. The data from the vendors product profile is also presented for comparison with the automation.

Questions:

- **Since RNA is very thermolabile and sensitive to degradation, did it withstand the entire process of automation?**
- **Did the workstation accurately dispense reagent and sample RNA volumes?**
- **Did the workstation properly mix (by pipetting) the reagents?**
- **Was there any possible cross-contamination, leading to possible ambiguous interpretation of the gel results?**

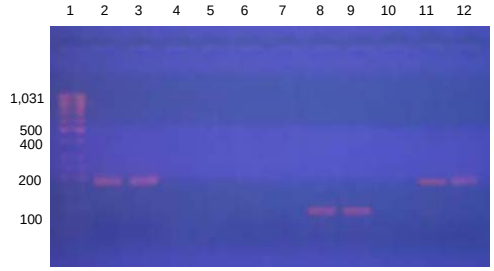
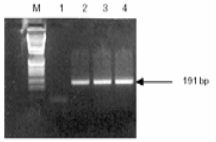


Figure 1. Ethidium bromide gel showing amplicons from the H5N1 detection kit. Gel, lane # 1 (DNA Ladder, 50bp-1 kb), lane 2-3 (Influenza type A amplicon 191bp), lane 4-5 (Negative control for A), lane 6-7 (Negative control for N1), lane 8-9 (N1 amplicon, 107bp), lane 10 (Negative control for H5), and lane 11-12 (H5 amplicon, 192bp).

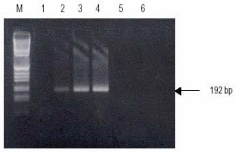
Answers: Figure 1 is a picture of the agarose gel showing the detection of amplicon bands specific to the H5N1 strain. The results indicate that the RNA withstood the process of automation that allowed its successful reverse transcription. The location of the bands of the respective amplicons in the agarose gel in relation to the 50 bp DNA ladder showed the presence of 107, 191, and 192 bp bands confirming the presence of viral traits for determining its identity. This data also suggests the successful reverse transcription of the viral RNA into cDNA in all the three separate reaction mixes. Absence of false positives and false negatives, reflects no cross-contamination.

Q 2. Are these results supported by any data from other sources?

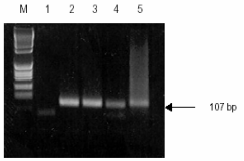
Answer (i): Reverse transcription and amplification with Rx-Mix A (presented by the kit vendor). Lane M (DNA standard), 1 (Negative control, dH_2O), 2 (Influenza A PR/H1N1), 3 (Patient serum), and 4 (Influenza A /H5N1).



Answer (ii): Reverse transcription and amplification with Rx-Mix N5 (presented by the kit vendor). Lane M (DNA standard), 1 (Negative control, dH_2O), 2 (Influenza A /H5N1, 10-2), 3 (Influenza A /H5N1, 10-1), 4 (Influenza A /H5N1), 5 (Influenza A PR /H1N1) , and 6 (Patient serum).



Answer (iii): Reverse transcription and amplification with Rx-Mix N1 (presented by the kit vendor). Lane M (DNA standard),1 (Negative control, dH_2O), 2 (Influenza A PR /H1N1), 3 (Influenza A /H5N1), 4 (Influenza A /H5N1, 10-1), 5 (Serum patient)



Q 3. Was the avian flu RNA extracted using a VERSA Workstation?

Answer: The avian flu RNA used in the present validation studies was supplied by the kit company as frozen vial. However, Aurora Biomed's VERSA Workstation for Nucleic Acid Extraction & PCR Setup is a dedicated platform for the extraction of genomic or cellular RNA.

VI. Conclusion

- VERSA Mini PCR Workstation is capable of automating processes involved with influenza virus H5N1 detection.
- The VERSA can be used for reverse transcription of positive control avian flu RNA (supplied by a kit) to synthesize cDNA.
- The VERSA can be used for PCR setup using cDNA of influenza A type, H5, and N1 amplicons and specific master mixes supplied in the kit.
- The amplification results were consistent and clean, indicating accurate and precise volume delivery of samples

VII. Acknowledgements

We are also thankful to Alicia Davis and Victor Navasero for technical help.

VIII. References

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3. Product manual: H5N1 Detection Kit for the detection of Influenza A/H5N1. Mediagnost, Germany.

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