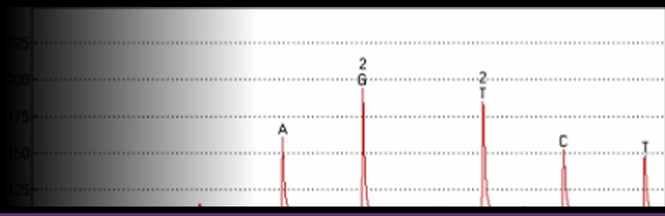
Evaluation of QIAGEN'S PyroMark Q48 Pyrosequencer



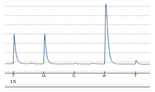
G. Stephanie Feumba, Jennifer Laplante, Kirsten St. George
Laboratory of Viral Diseases, Wadsworth Center, Albany, NY

NEW YORK STATE | Department of Health | Wadsworth Center

Background

- Pyrosequencing is a short-read sequencing method that relies on the generation of light signal from the release of pyrophosphate when nucleotides are incorporated during the sequencing process.
- Pyrosequencing has been used in New York State (NYS) for influenza antiviral resistance surveillance for more than 15 years. The method can be used in a high throughput capacity, with rapid results, and is much less expensive than whole genome sequencing.
- Qiagen is the only supplier of pyrosequencing instrumentation. This validation was prompted by the decommissioning (end-of-life status) of the currently used pyrosequencing instrument, the PyroMark Q96ID (Q96, Ref.1). The suggested replacement system is the PyroMark Q48 (Q48).
- The Q48 is advertised to produce the same results as the Q96 with less technician manipulation and reduced processing time. The Q48 is smaller in size and can only process 48 samples per batch.
- As the sole support laboratory to the CDC for antiviral resistance testing for influenza, it is imperative that the NYS public health laboratory maintain full capacity for all analytical methods, and therefore must transition to replacement instruments as they become available. This allows NYS to continue to provide crucial influenza antiviral susceptibility profiling.

Workflow

Step 1	Step 2	Step 3	Step 4
Extraction	PCR	Sequencing	Result Analysis
			
https://www.biomerieux-usa.com/clinical/emag	https://www.fishersci.com/shop/products/simpliamp-thermal-cycler-extended-warranty-package-5/A24811	https://www.qiagen.com/us/products/discovery-and-translational-research/pyrosequencing/pyromark-q48-autoprep-and-accessories	This image was generated with data from the Virology Laboratory, Wadsworth Center.
110µL of total nucleic acid from 350µL of residual patient samples or cultured isolates was obtained using the Biomerieux eMAG instrument	Conventional RT-PCR was performed on a SimpliAmp instrument, using QIAGEN One-step kit and primers from CDC (Ref.2)	Sequences were obtained with QIAGEN PyroMark Instruments using primers, PCR-generated amplicons, and PyroMark reagents	Analysis of pyrograms includes reviewing sequences and signal intensity.

Method & Materials

- Validation of the Q48 against the Q96 was performed with pyrosequencing influenza A antiviral resistance mutation detection assays for mutations at codons 275 and 223 on A/H1pdm09, and those at codons 119, 292, and 294 on A/H3N2 (Ref. 2), all of which are routinely used in the NYS surveillance program.
- Residual portions of original patient material containing the viruses, or cultured isolates, were extracted on an eMAG automated nucleic acid extractor for all experiments.
- 10-fold serial dilutions of cultured isolates of A/H1pdm09 and A/H3N2 RNA were tested by real-time RT-PCR to obtained Ct values. Conventional RT-PCR was performed simultaneously.
- Conventional RT-PCR amplicons were prepared for sequencing on the Q96ID and Q48. Each instrument requires different reagents and sequencing primer concentration.
- The initial side-by-side experiment compared sequences generated from A/H3N2 positive samples previously sequenced on the Q96ID to established a baseline read between the two instruments using the default settings for the Q48.
- RT-PCR products from experiments were analyzed by gel electrophoresis to confirm the presence of amplicons and fragment size when troubleshooting failed sequencing reactions.
- Additionally, a QIAGEN field application specialist (FAS) was contacted to assist with instrument troubleshooting and investigations for failed sequencing on the Q48.
- Multiple modifications were investigated to enhance and optimize the conventional RT-PCR and sequencing reactions including:
  - Addition of Q solution to the master mix for increased amplicon yield.
  - Annealing temperature gradient to confirm optimal temperature for RT-PCR.
  - Adjusted amplicon concentration for sequencing using Millipore spin column.
  - Variable volumes of magnetic beads for the sequencing reaction.
- Finally, sequencing was also attempted on another Q48 instrument

Q48 Anticipated Advantages

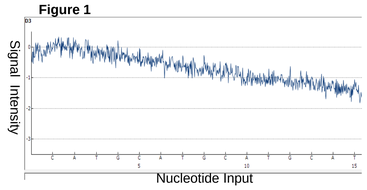
- Q48 automated protocol requires less manual interaction: which should decrease testing turnaround time.
- Improved workflow with touch screen display that guides users through the process.
- Compact instrument size: Q48 require less bench space compared to the Q96.
- Q48 enabled for network connectivity. Q96 runs on Windows XP which is no longer supported.
- Potential availability of "advanced reagents" for longer read lengths on the Q48.
- Additionally, QIAGEN advertises that data is comparable, if not better, to that produced by Q96.

Results & Observations

The LOD for A/H1pdm09 and A/H3N2 were tested across Ct ranges of 13-37 and 16-37 respectively. The Q96 produced sequence on samples up to Ct 33 and 29 respectively, while the Q48 produced no sequence on any samples.

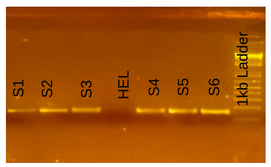
Figure 1 is an example failed pyrogram for the 275Y assay on A/H1N1 on the Q48, representative of results from Q48 sequencing for all mutations on both A/H1N1 and A/H3N2 assays. The sequences obtained from the first side-by-side experiment were inconclusive. The Q96 produced clear sequences, appropriate for the expected target. The Q48 did not generate any acceptable sequence and the pyrograms looked like noise.

Figure 1



Due to repeated failed sequencing experiments on the Q48, presence and size of amplicon was confirmed by gel electrophoresis. **Figure 2** shows bands from six samples (S1 to S6) of residual A/H1N1 amplicon, and a negative control (HEL), tested by gel electrophoresis. The amplicon sizes (154bp) were confirmed using 1kb ladder.

Figure 2



Troubleshooting experiments included, adding QIAGEN's Q solution to the conventional RT-PCR recipe. Attempts at concentrating the RT-PCR amplicons did not improve the sequences produced on the Q48. Additionally, modifications to the RT-PCR annealing temperature, from 52°C to 58°C, did not improve the quality of the pyrograms. Pyrograms generated from Q48 were inconclusive and included multiple false peaks (**Figure 3a**), while pyrograms from the Q96 (**Figure 3b**) were clear and showed appropriate peaking for the expected target.

Figure 3a

A(H3N2)-NA119 _ Q48

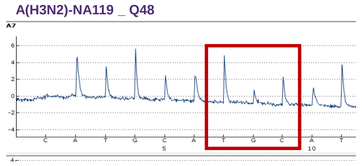
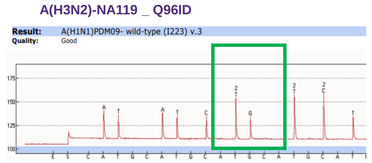


Figure 3b

A(H3N2)-NA119 _ Q96ID



The Q48 performed unpredictably, with frequent failed experiments, and a new Q48 was acquired to continue the validation. The first promising results were generated on this instrument using RNA template from an influenza A/H1N1 cultured isolate, and a new cartridge with enzyme injector. The resulting pyrograms were correct for the targeted genome, yet the individual nucleotide intensity was low (**Figure 4a and 4b**).

Figure 4a

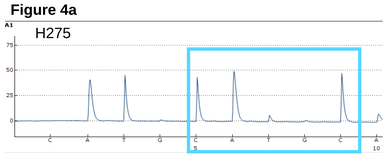
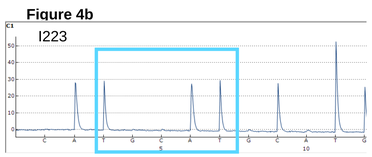


Figure 4b



Results & Observations

The one successful experiment was repeated 4 times using RNA extracted from residual patient samples and the results were continuously unacceptable. Sequences generated from the Q48 were very weak, not clear and included many spurious peaks (**Figure 5a**), while those obtained from the Q96 reliably passed (**Figure 5b**).

Figure 5-a PyroMark Q48

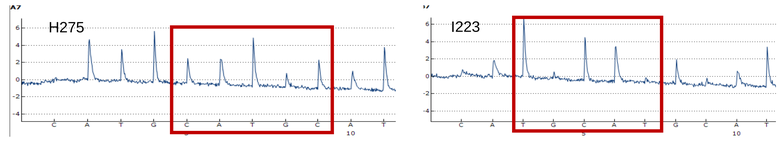
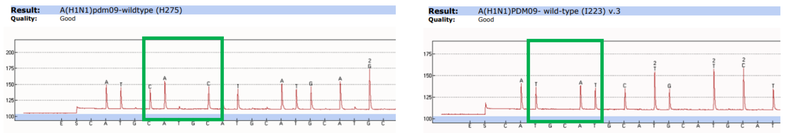


Figure 5-b PyroMark 96ID



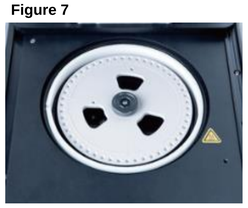
Numerous performance issues were encountered with the Q48. The injector responsible for the dispensation of enzyme in the sequencing reactions (**Figure 6**), failed frequently and unexpectedly. This has since become a well-known issue among users and the manufacturer (personal communications). The Q48 produced pyrograms after a new injector cartridge was obtained and installed (for enzyme dispensation). However, the data was unacceptable, the nucleotide signal intensity was very low and there were many spurious peaks as represented in Figure 3a.

Image taken from QIAGEN's PyroMark Q48 user manual page 51
(Figure 6)

Figure 6

The Q48 also has a few problematic design and processing issues, for example, the design and performance of the Q48 disk. The RT-PCR product is manually loaded on each of the 48 wells of the disk. Following template addition, the Q48 automates the pyrosequencing process. The process of pipetting clear, small volumes into the opaque small wells of the disk, without the ability to use a multichannel pipette, can be technically very difficult and is highly error prone. Additionally, the lid of the sequencer remains open, leaving the disk exposed, as shown in **Figure 7**, during the 38-minute "warm-up" period preceding the pyrosequencing, exposing the disk to risk of air drafts and cross-contamination across the wells.

Figure 7



Manual preparation and sequencing on the Q48 takes approximately 116 minutes and includes ~30 minutes to prime the instrument with reagents, 15 minutes for template and magnetic beads addition, 38 minutes for the disk warm-up step, and 33 minutes for pyrosequencing. Alternatively, from the preparation to obtaining sequence results, the Q96 only requires ~70 minutes.

Conclusion

Ultimately, only 1 of the total of 38 validation experiments conducted using well established A/H1pdm09 variant assays produced acceptable sequence data.

The only successful pyrosequencing run on the Q48 was completed using extracted RNA from highly concentrated cultured virus isolates. However, even these results were not reproducible on extracted RNA from residual patient samples, with resulting pyrograms consisting of a series of spurious peaks.

The PyroMark Q48 did not perform as expected and is not an acceptable replacement to the PyroMark Q96ID

References

1. https://www.qiagen.com/us/products/discovery-and-translational-research/pyrosequencing/pyromark-q96-systems
2. Deyde VM, Okomo-Adhiambo M, Sheu TG et al. Pyrosequencing as a tool to detect molecular markers of resistance to NAIs in seasonal influenza A viruses. Antiviral Res.81(1), 16–24 (2009).