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OBJECTIVES. An increasing number of oseltamivir-resistance associated mutations (H275Y in the neuraminidase NA gene) are being reported in H1N1/2009 viruses isolated from patients treated with oseltamivir, particularly in the immunocompromised.¹ It is important to identify the presence of these drug resistance-associated mutations to optimize the care for such patients.

METHODOLOGY. A rapid and cost-effective High Resolution Melting (HRM) PCR assay was developed for this purpose using an unlabeled probe to screen for the H275Y mutation among patients treated with oseltamivir, especially the highly susceptible immunocompromised patients. Isolated probe PCR (IP-PCR)² was performed using Superscript III Platinum One-step qRT-PCR kit (Invitrogen) on either the LightScanner LS32 (Idaho) or the LightCycler LC v2.0 (Roche) system, both of which utilize the uniquely designed LC glass capillaries as reaction receptacles. To initiate the IP-PCR, 2 μ L of extracted viral RNA was added to 6 μ L of reaction mix containing optimized amounts of forward and reverse primers. A calculated excess amount of forward primer and unlabelled probe mix (2 μ L) was then aliquotted into the upper well of the capillary tube, without being spun down with the reaction mix. Upon completion of the IP-PCR, the 8 μ L reaction mix was mixed with the pre-deposited primer/probe mix by inversion and reverse-spinning, followed by an "extended" 15-cycle asymmetric amplification reaction and continuous HRM data acquisition. The *in vitro* synthesized RNA transcripts for both wildtype and mutant were diluted using pooled negative RNA extracts and used as the standard calibrator. The lower limit of detection (LLOD) and lower limit of quantification (LLOQ) for the mutant strain were determined. To estimate the proportion of H275Y mutant population in a mutant/wildtype mixture, a series of 0-100% mixed-mutant/wildtype standards were tested, at 10% incremental intervals and 10⁵ RNA copies/reaction.

RESULTS. The melt peaks for the unlabeled probe with a mismatched base C (wildtype) and that with a perfectly matched base T (mutant) were clearly discernable at 65.5°C and 69.25°C (Figure 1), respectively. The assay reproducibly amplified concentrations of 20 to 2x10⁷ RNA copies/reaction, thus giving an LLOD of 20 copies/reaction. The LLOQ, defined as the lowest concentration whereby the difference of 3.33 Ct value per log is maintained, was 200 copies/reaction (Figure 2). Good resolution of the melt peaks was obtained for the whole series of 0-100% mixed-mutant/wildtype standards (Figure 1).

CONCLUSION. This assay is able to provide easily discernable results for qualitative and quantitative detection of WT (H275)/mutant (Y275) mixes. The preliminary results suggest that this assay and platform may accurately identify H275Y populations down to 10% of the viral population, though further validation on clinical specimens is required.

REFERENCES & ACKNOWLEDGEMENTS

1. *Wkly Epidemiol Rec* 2010;85:37-40.
2. *Biotechniques* 2007;43:87-91.

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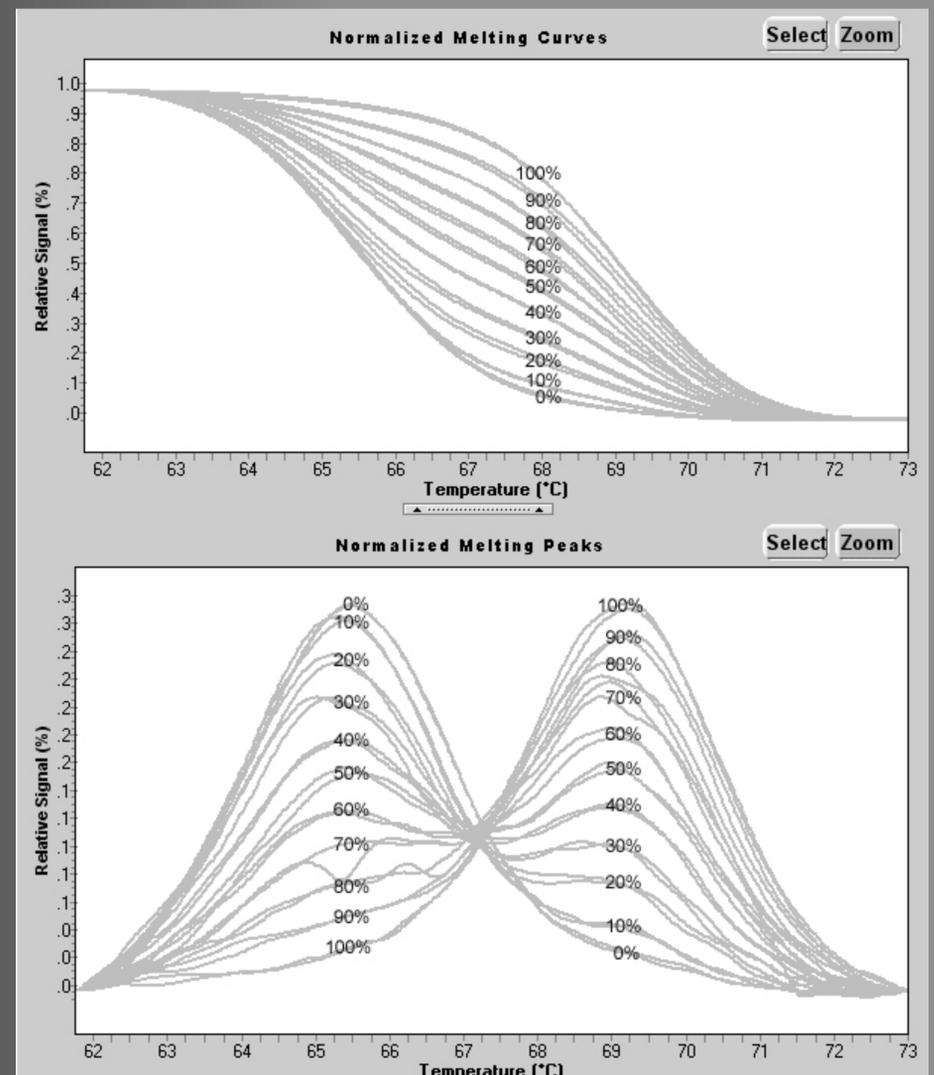


Figure 1. Normalized melting curves and peaks for a series of 0-100% mixed-mutant/wild type standards, at 10% incremental intervals and 10⁵ copies/reaction total *in-vitro* RNA transcripts concentration. The melting peaks for the base C (wildtype) and base T (mutant) were distinguishable at 65.5°C and 69.25°C, respectively.

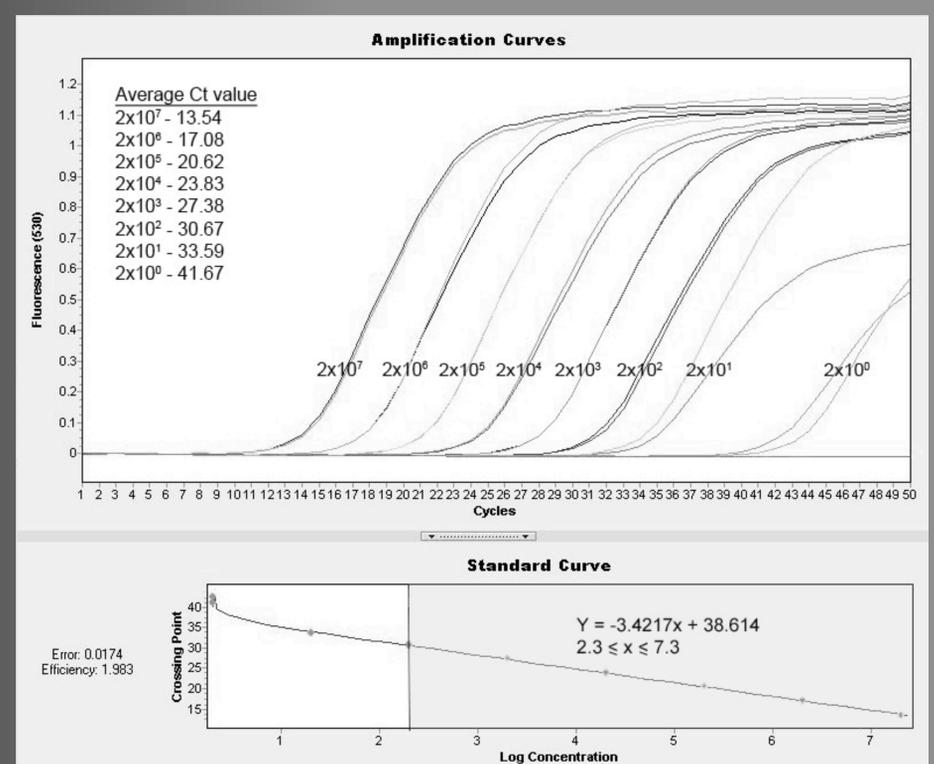


Figure 2. Amplification curves and standard curve of duplicate RNA transcript standards at 2x10⁰– 2x10⁷ copies/reaction. Ct value differences closed to 3.33 were maintained between 2x10²– 2x10⁷ copies/ reaction. Linear equation was derived from the standard curve at value 2.3 ≤ Log concentration/x ≤ 7.3 (Shaded), with m = -3.4217. The PCR efficiency calculated using the LC v2.0 software was 1.983.