



# Rapid Detection and Analysis of Subtypes of Avian Influenza in a Single Tube Using LATE-PCR

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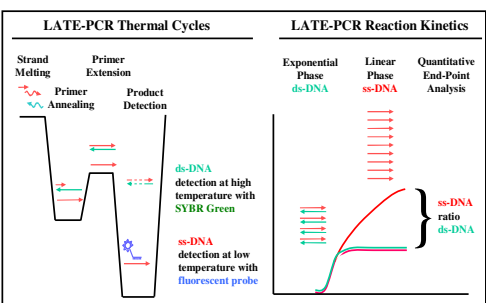
(email: [Wangh@brandeis.edu](mailto:Wangh@brandeis.edu), or Google: LATE-PCR)



## Introduction

Pathogenic strains of avian influenza, particularly subtypes H5 and H7 continue to spread and evolve through out Eurasia and Africa, causing death and requiring culling of millions of birds. Several hundred people have died of avian influenza primarily following direct contact with infected poultry, but there is reason to fear that relatively few genetic changes could lead to efficient human-to-human transmission raising the specter of a devastating global pandemic. Rapid, accurate detection of the presence and type of influenza in people, birds, and other species is urgently needed, particularly in remote rural areas where people live very close to their livestock. In collaboration with Smiths Detection, Inc. (Watford, U.K.) we are using two novel technologies developed in our laboratory at Brandeis University, LATE-PCR (U.S. Patent 7,198,897) and PrimeSafe™ (a mis-priming preventing compound), to construct a highly multiplexed assay that can detect and distinguish between several subtypes of avian influenza and their strains in a single tube. This assay is designed to work on a battery-powered point-of-care instrument, the BioSeeq, which is being designed and engineered by Smiths Detection, Inc.

## The Logic of LATE-PCR

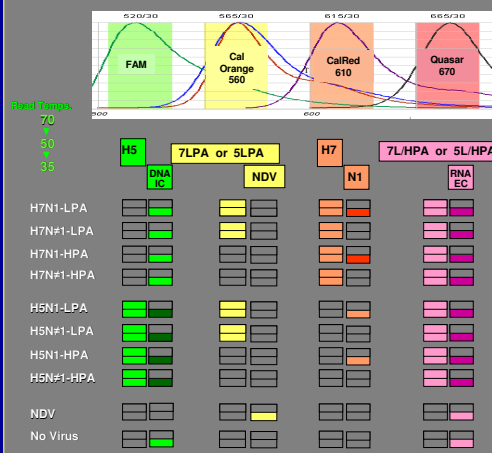


LATE-PCR is an advanced, efficient form of asymmetric PCR which utilizes an Excess Primer and a Limiting Primer having a higher melting temperature ( $T_m$ ). Phase I amplification results in exponential amplification of a limited number of double-stranded amplicons. Phase II amplification uses one strand of the double-stranded amplicon to linearly generate single-stranded amplicons. The single-strands accumulate to up to 10-20 fold more abundant than the double-strands. Single-stranded amplicons can be detected after the extension-step of the reaction, or at end-point, using low- $T_m$  probes.

## Assay Design (Full Assay)

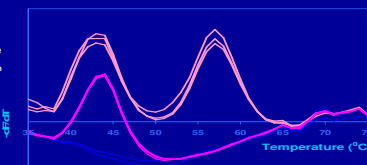
### Assay Components and Properties

- Four Fluorescent Dyes
- Seven Pairs of Primers in the Multiplex
- Ten Low- $T_m$  Probes (two sets: four bind at 35°C, six at 50°C)
- One Internal (DNA) and one External (Armored RNA) Control
- Read Only at End Point
- Read at 70°, 50°, 35°C (calculate 50/70°C and 35/70°C ratios)

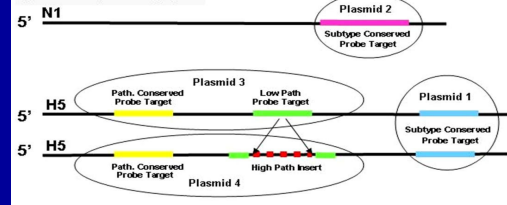


Probes with the Same Fluor and Different  $T_m$  in the Multiplex:

Two Quasar670 Probes  
Higher  $T_m$ : HS LPA/HPA  
Lower  $T_m$ : Ext. Control



## Assay Development (H5N1)

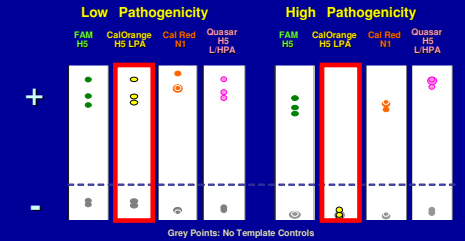


Four plasmids containing different parts of the AI genome were constructed and transcribed *in vitro* using T7 polymerase. Plasmids 1 and 2 contained conserved regions of the H5 and N1 genes. Plasmids 3 and 4 contained a conserved region and either a Low Pat. (probed) or a High Pat. (not probed) region.

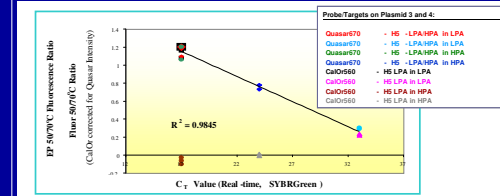
## Experimental Results

Sets of three transcripts were combined in equal concentrations to simulate the RNAs found in two different viral strains: H5N1 Low Pathogenic and H5N1 High Pathogenic. Both these sets of three RNAs were then mixed with three pairs of primers. One primer in each pair primed reverse transcription of the corresponding cDNA, after which the second primer in each pair primed second strand synthesis. Four sequence-specific probes, one for each of the possible DNA targets were also present in the reaction. The results show that the H5 and N1 amplicons were generated in both reactions. The amplicon covering the pathogenicity region of the H5 gene was also generated in both cases. But, as expected, the Low Path. amplicon hybridized to both probes for this region, while the High Path. amplicon only hybridized to the probe for the conserved sequence within the pathogenicity region. Tetraplex RT-LATE-PCR assays including an EC RNA were also successful.

## Alternate Outcomes of the RT-LATE-PCR Multiplex End Point Assay for H5N1 Avian Influenza



## Quantitative Correlation Between End-Point Detection of Targets in a Triples RT-LATE-PCR and CT Values of Single Targets in Real-Time PCR



## Conclusions

Once this assay is fully implemented on the Smiths Detection BioSeeq instrument, sample preparation and addition of all reagents will occur within a sealed canister which sits atop the thermal cycler. We estimate that the total elapsed time from sample-to-result will be two hours or less. Finally, because of the nature of the LATE-PCR amplification process, each of the single-stranded amplicons generated in a multiplex reaction of the type described here can be used for Dilute-N'-Go dideoxy-sequencing which is both convenient and cost-effective. This is particularly important in the case of RNA viruses like Avian Influenza in which sequence changes occur readily and can significantly alter the infectivity and evolution of the virus.