

LATE-PCR for Detection and Analysis of Infectious Diseases

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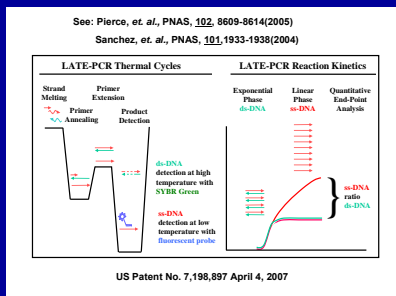
(email: Wanhg@brandeis.edu, or Google: LATE-PCR)



The Logic of LATE-PCR

LATE-PCR, invented in our laboratory, is an advanced form of asymmetric PCR that uses two primers of unequal concentration and differing melting temperatures to generate double-stranded DNA amplicons exponentially, followed by linear amplification of one strand of each amplicon. One of the two primers can also be used to synthesize cDNA from RNA prior to the start of amplification, thus allowing for RT-PCR. PrimeSafe™, also invented in our laboratory, is a PCR additive that enhances construction of single-tube multiplexed assays comprised of several target sequences from a bacterial or viral genome. These assays can detect as little as a single DNA or cDNA molecule. The resulting single-stranded DNA amplicons are either quantified at end-point by use of multiple sequence-specific probes, or mis-match tolerant probes, or via our convenient Dilute-'N'-Go sequencing protocol.

We are employing these technologies to detect and analyze animal and human infectious diseases in the laboratory or in the field using the BioSeq, a novel point-of-care device developed by Smiths Detection, Inc. Our Avian Influenza assay, for instance, detects both low/high pathogenic variants of both subtypes H5 and H7 and their N subtypes, plus Newcastle Virus, if Avian Influenza is not present. An internal DNA standard and an external RNA control, guard against false negatives. Depending on which virus is present, sets of 1-3 single-stranded cDNA target sequences are amplified and detected based on their patterns of hybridization to ten fluorescent probes in four colors.



PrimeSafe

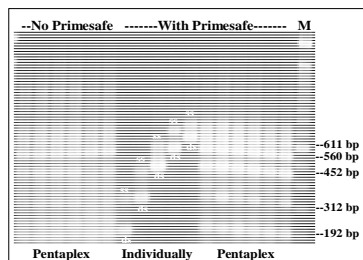
PrimeSafe™ Suppress All Forms of Mis-Priming

- Primer Dimers
- Errors during Sample Prep.
- Errors during Exponential Amplification
- Errors during Linear Amplification
- Errors during Multiplexing

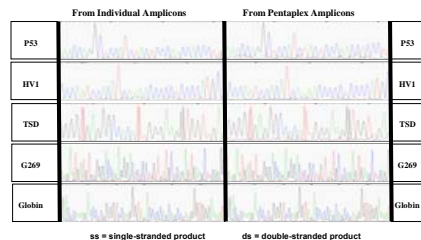
PrimeSafe™ is available from Smiths Detection
Email blodetection@smithsdetection.com

Multiple forms of mis-priming can occur in both conventional and LATE-PCR amplification reactions. Hot-Start only prevents errors that occur before amplification begins. PrimeSafe, a new reagent invented in our laboratory suppresses all forms of mis-priming. Multi-plexing is an invitation to mis-prime, but addition of PrimeSafe makes multi-plexing easy, see below.

LATE-PCR Multi-plexing



Dilute-'N'-Go Sequencing



Dilute-'N'-Go Sequencing of the Limiting Primer Strand can be carried out directly from a Multi-plex LATE-PCR assay by simply adding a different sequencing primer to each aliquot of the reaction. The resulting sequences are directly comparable to sequences obtained from single-plex LATE-PCR reactions. Using this approach we have amplified and sequenced as many as 15 different products in a single LATE-PCR assay. The upper limit of complexity using this approach remains to be established. Using this approach it is possible to amplify many sequences in a LATE-PCR, some of which are detected immediately and some of which are identified later by sequencing.

The BioSeq: A Portable Device



The instrument contains five integrated Sample Preparation and PCR stations, allowing samples to be extracted, nucleic acid purified and PCR to be carried out without any user intervention. Up to five samples can be processed simultaneously. Each sample can be running a different profile or the same one and the analysis can be started at the same time or at different times, for example, as each sample is collected.

The instrument is a ruggedised 4 colour, actively cooled PCR instrument similar in capability to a lab instrument. However, this instrument is battery powered and can be decontaminated by immersion in 2% Virkon. It is designed to be used in adverse weather conditions.

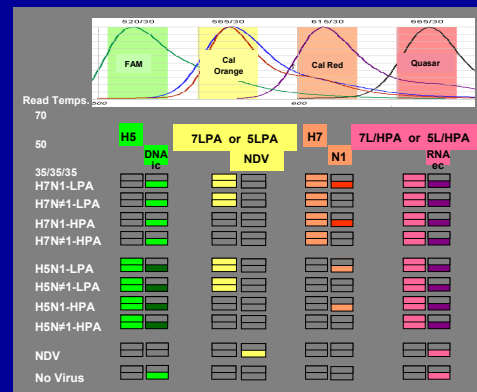
The system has built-in wireless communications and GPS so that the instrument can send the results of the analysis and the location to a central data collection system for real time control of an outbreak.



RT- LATE-PCR for Avian Flu

Assay Components and Properties

Four Fluorescent Colors
Read at 70°, 50°, 35° C
Seven Pairs of Primers in the Multiplex
Ten Probes in the Multiplex
Read Only at End Point



H7N1 Low Pathogenicity
H7 not N1 Low Pathogenicity
H7N1 High Pathogenicity
H7 not N1 High Pathogenicity

H5N1 Low Pathogenicity
H5 not N1 Low Pathogenicity
H5N1 High Pathogenicity
H5 not N1 High Pathogenicity

Newcastle Virus

No Virus, system ok

Conclusions

Four different plasmids containing different parts of the Avian Influenza genome were constructed and transcribed *in vitro* using T7 polymerase. Sets of three transcripts were combined in equal concentrations to simulate the mixtures of 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.

Once this assay is fully implemented on the Smiths Detection BioSeq instrument, sample preparation and addition of all reagents will occur within a sealed canister which sits atop the thermal cyclor. We estimate that the total elapsed time from sample-to-result will be less than one hour.

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.

Rice et al. (2007) Nature Protocols, vol. 2, #10, 2429-2438
Additional LATE-PCR publications are available at:
<http://www.brandeis.edu/projects/wanhg/lab/>