



# LATE-PCR Assays for Point of Care Detection and Analysis of Veterinary Diseases

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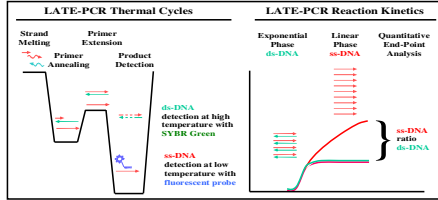
## Introduction

There is a growing need in veterinary and human medicine for diagnostic assays that can rapidly detect and analyze numerous species and strains of infectious organisms and viruses. RNA viruses are particularly challenging because they evolve rapidly. In the case of veterinary medicine, tests have to be done at pen-side or in the field and the findings can have great economic impact, including slaughter of many animals. Currently the use of RT-PCR is limited in scope and few, if any, assays provide results from multiplex data in the field. We have overcome these limitations by constructing assays based on LATE-PCR and (RT)-LATE-PCR. We are collaborating with Smiths Detection to implement these assays on an automated portable sample preparation and PCR system.

## 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 amplicon. 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 that are either sequence specific or mis-match tolerant.

See: Pierce, *et. al.*, PNAS, **102**, 8609-8614(2005)  
Sanchez, *et. al.*, PNAS, **101**, 1933-1938(2004)

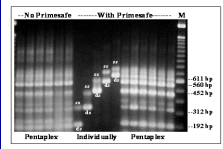


US Patent No. 7,198,897 April 4, 2007

## PrimeSafe & Multiplexing

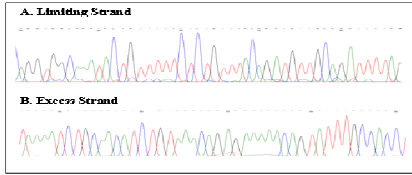
PrimeSafe™ Suppress All Forms of Mis-Priming

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



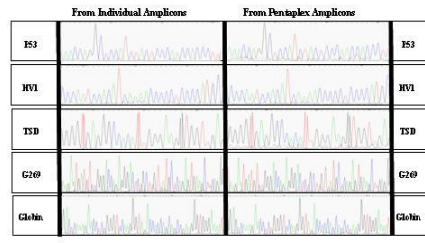
## Dilute-'N'-Go Sequencing

Both the single strand generated in LATE-PCR and the template strand from which it is generated can be sequenced by direct dilution into a dideoxy sequencing mix without clean-up.



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.

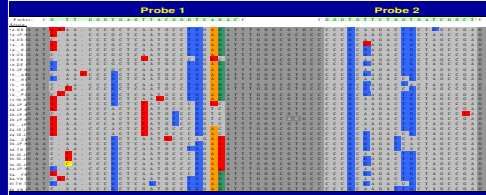
## Sequencing All Amplicons in a Multi-plex



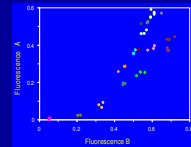
Dilute-'N'-Go Sequencing of the Limiting Primer Strand can be carried out directly from a Multi-plex LATE-PCR assay by simply adding a different sequencing primer to each aliquot of the reaction. The resulting sequences are directly comparable to sequences obtained from single-plex LATE-PCR reactions. 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.

## Multi-probing

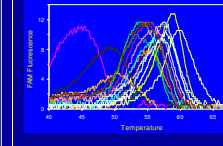
The single-stranded products of a LATE-PCR assay can be detected in real-time or at end-point using mis-match tolerant probes. Mis-match tolerant probes hybridize to both perfectly complementary and partially-complementary target sequences as a function of temperature. In LATE-PCR assays the probes hybridize to their targets over a broad range of temperatures which are all below the annealing temperature used for primers. Thus, mis-match tolerant probes do not decrease the efficiency of amplification. As shown below, two mis-match tolerant probes can hybridize to two different variable sequences within a single target molecule. Each probe melts off the target at its own temperature, depending on the extent of complementarity. Fluorescent ratios can be constructed from the resulting data and these ratios can generate a Fluorescent Signature, characteristic of the particular sequence of the target strand.



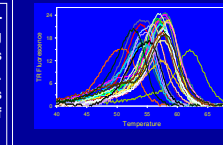
### Fluorescence Signatures



### Probe 1 - Target Melting Profiles



### Probe 2 - Target Melting Profiles



## 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.

## Abstract

Linear-After-The-Exponential (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. PrimeSafe™, also invented in our laboratory, is a PCR additive that suppresses mis-priming throughout all thermal cycles. PrimeSafe™, therefore, enhances construction of multiplexed LATE-PCR assays allowing simultaneous detection and analysis of several target sequences from the same bacterial or viral genome in a single tube. Such assays can detect as little as a single DNA or cDNA molecule. The resulting single-stranded DNA amplicons can be 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. Thus, these new platform technologies are more informative than conventional PCR and are ready for widespread use in many fields.

These tests can be carried out in the laboratory or in the field on the BioSeq, a novel point-of-care device developed by Smiths Detection, Inc.