**Background:** A false positive or false negative smallpox warning would also have severe consequences. This report describes a novel PCR-based orthopox assay that is sensitive, accurate, and able to distinguish among orthopox viruses.

**Methods:** Synthetic DNA targets were amplified using Linear-After-The-Exponential (LATE)-PCR, an advanced form of asymmetric PCR that efficiently generates single-stranded DNA. Probe hybridization to the accumulated single strands was analyzed at the end of amplification. The pair of LATE-PCR primers amplified a portion of the E9L Polymerase gene containing a conserved sequence present in all orthopox viruses and a second sequence that differs between Variola (spx), monkeypox (mpx), and various strains of cowpox (cpx). Two fluorescent probes hybridize to these sequences below the primer annealing temperature. Fluorescence detection was carried out at multiple temperatures after 60 thermal cycles to identify and quantify the amplification products. The assay also includes an internal control target that hybridizes to the conserved-organism probe, but at a lower temperature than the orthopox targets.

**Results:** Spx, mpx, and different cpx targets were amplified and measured at end-point. Fluorescence from the conserved-region probe was proportional to initial target concentrations and all viruses displayed the same melting profiles. In contrast, the melting profile of the variable-region probe was specific to the sequence of the target. The calculated fluorescence ratio of the two probes reduced variability among the melting profiles of replicate samples. Samples without orthopox targets exhibited fluorescence from the conserved-region probe, but only at low temperatures.

**Conclusions:** Quantitative end-point LATE-PCR and melt analysis can be used to distinguish different orthopox sequences. Each virus displays its own "fluorescent signature" when the ratio analysis is employed. These methods are of general utility for detection and resolution of both naturally occurring and genetically engineered pathogens.

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**LATE-PCR References**

LATE-PCR is an advanced form of asymmetric PCR which utilizes an Excess Primer and a Limiting Primer having a higher melting temperature (Tm). Phase 1 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 up to 10-20 fold more abundantly than the double-strands. Single-stranded amplicons can be detected using probes that are either sequence specific or mismatch tolerant.