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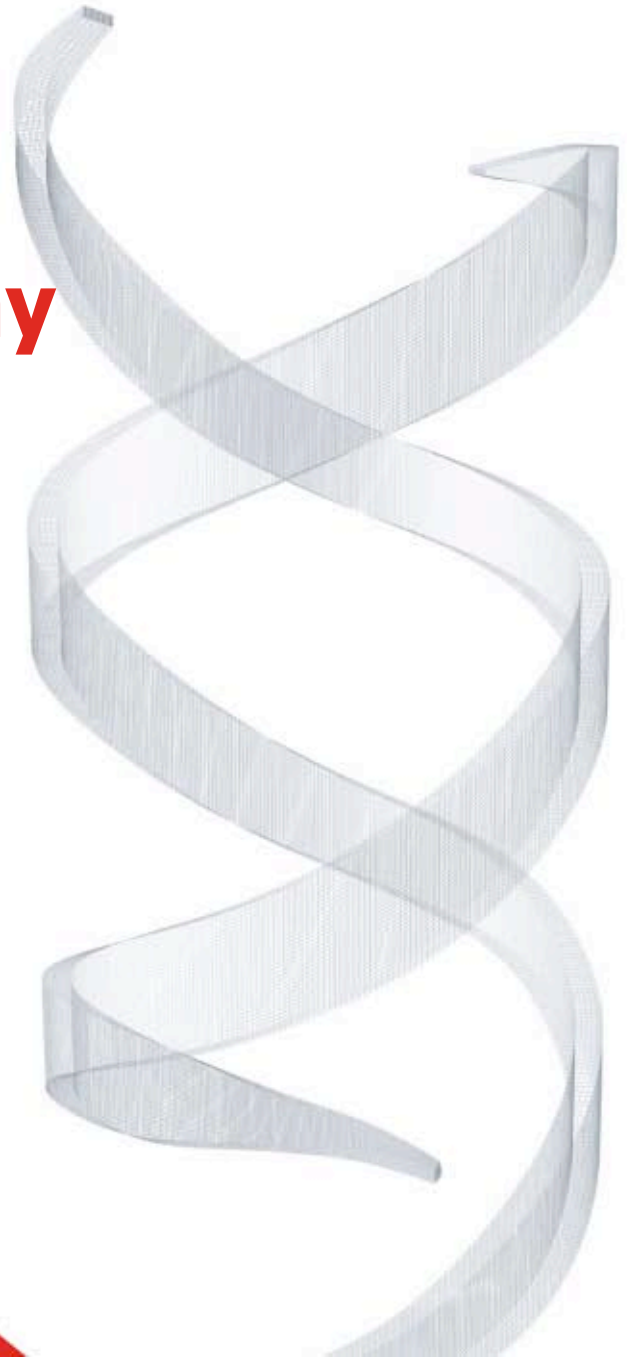
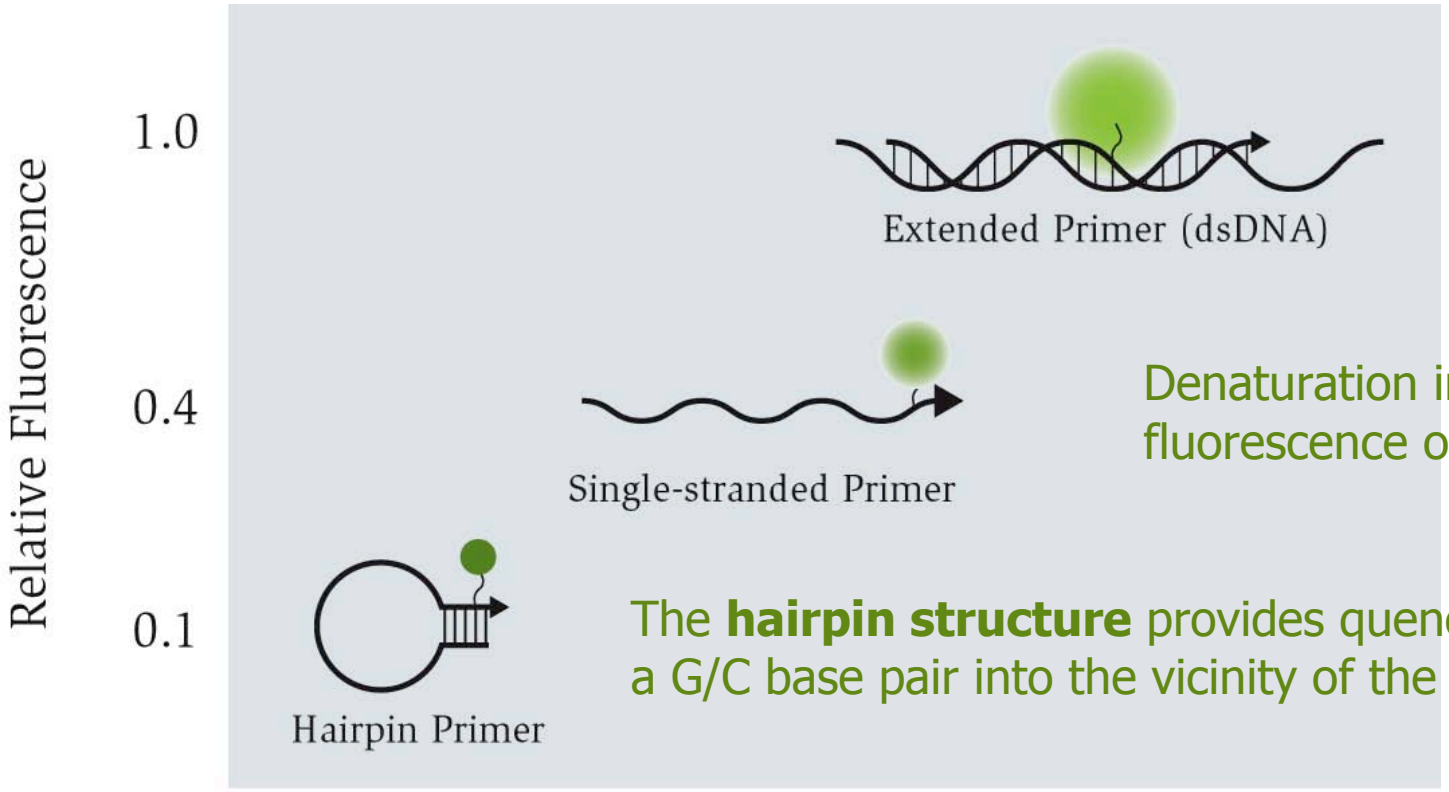
Two-color Multiplex Assay for the Identification of Orthopox Viruses with Real-time LUX™ PCR

Mark Andersen
Sandrine Javorschi-Miller
qPCR 2005



Standard LUX™ (Light Upon eXtension)



Extension further increases signal

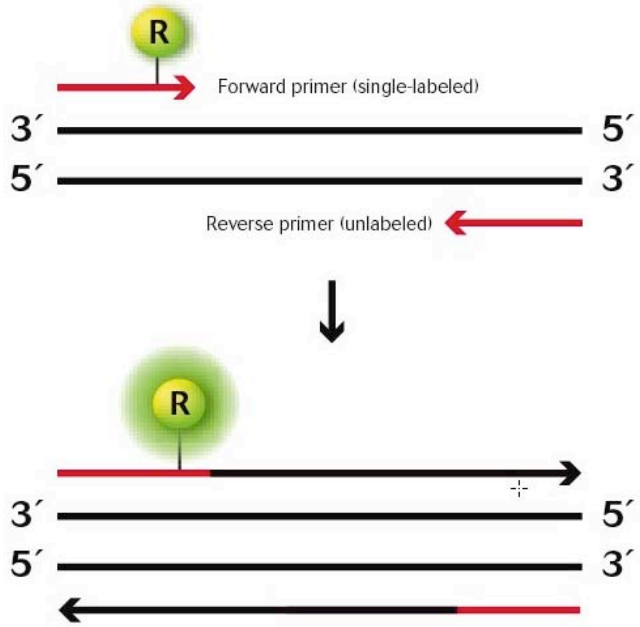
Denaturation increases the fluorescence of the primer

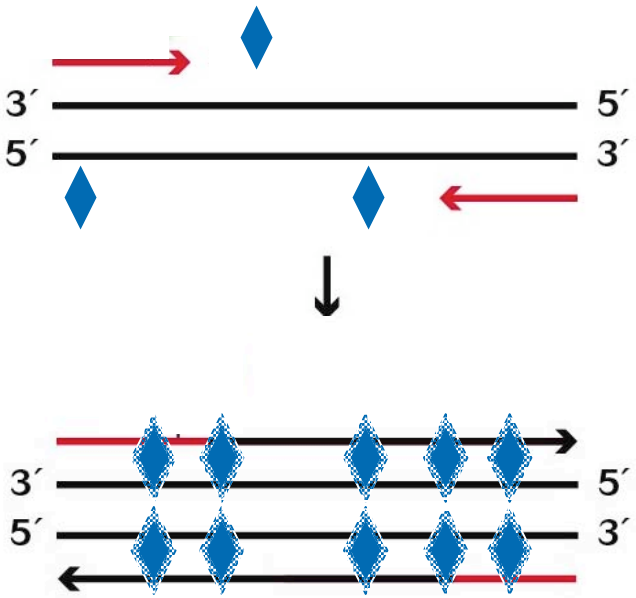
The **hairpin structure** provides quenching by bringing a G/C base pair into the vicinity of the flourophore

LUX primers have a fluorophore near the 3' end and a short complementary tail on the 5' end

Primer extension increases fluorescence

LUX™ and SYBR Detection

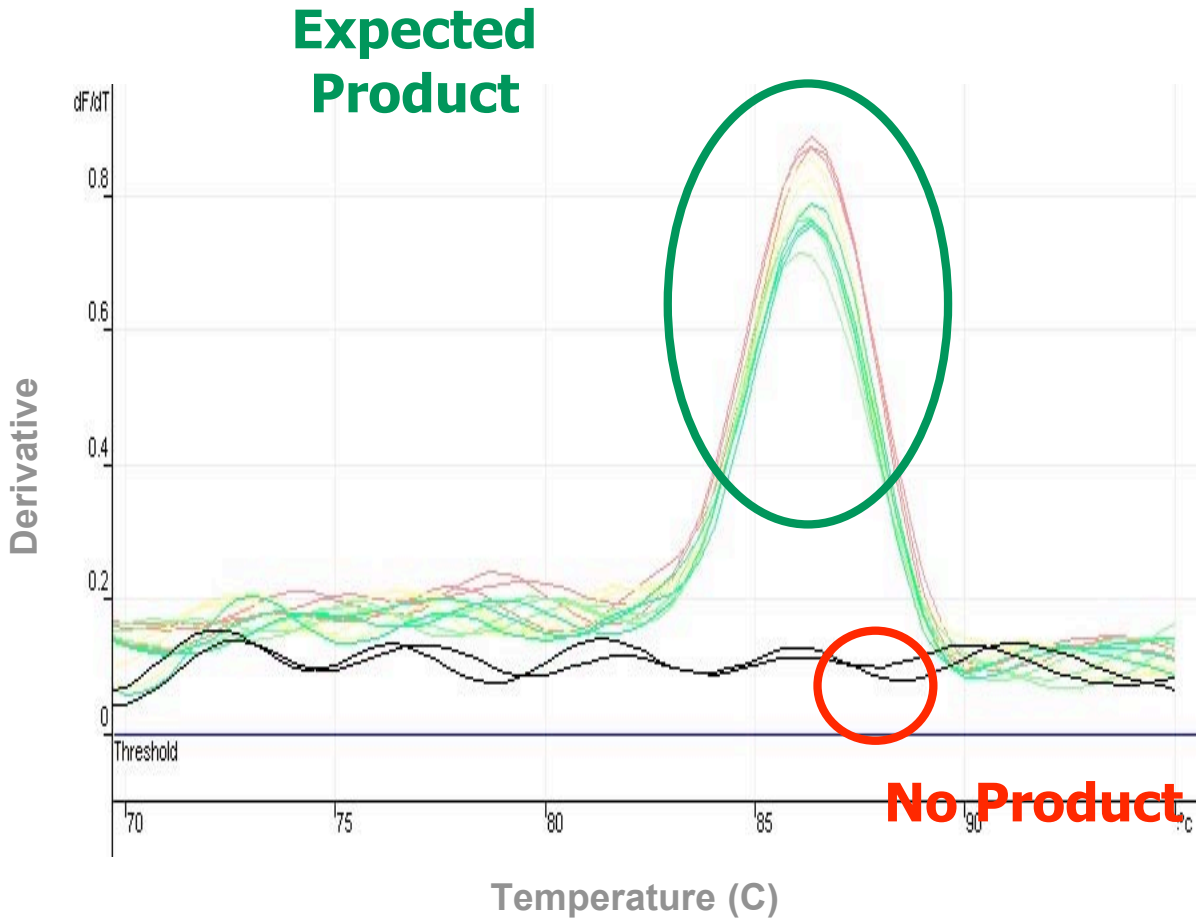




LUX **SYBR Green I**

Both chemistries label all extension products

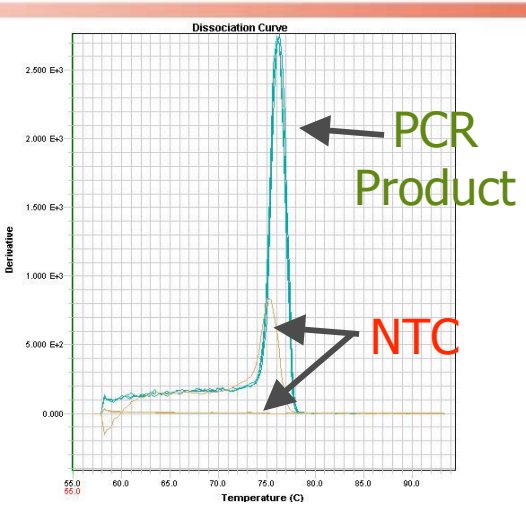
Dissociation Analysis

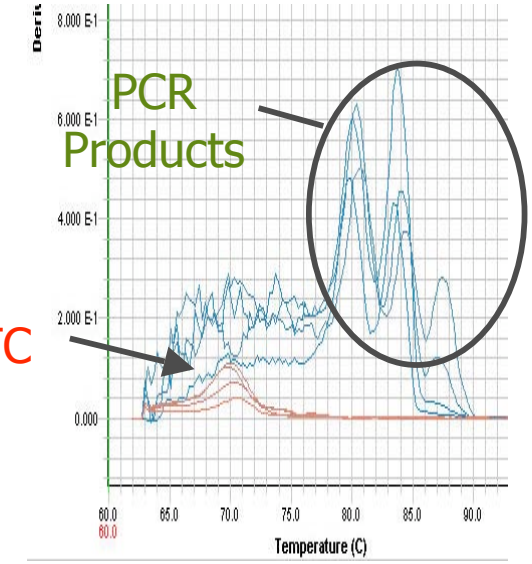


Verification of amplicon Tm

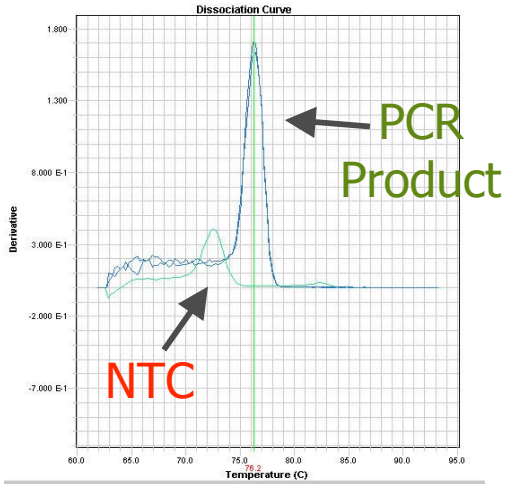
Dissociation Analysis with SYBR

Contamination
(NTC has same Tm as target)





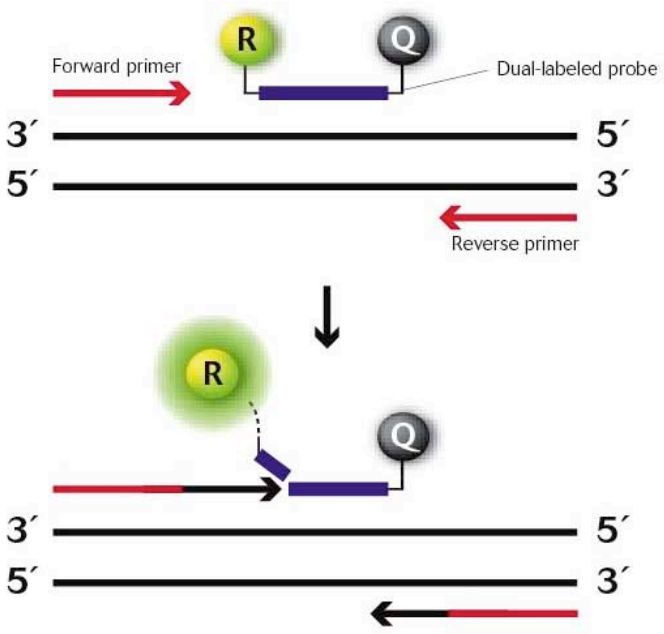
Nonspecific product
(different Tm than target)



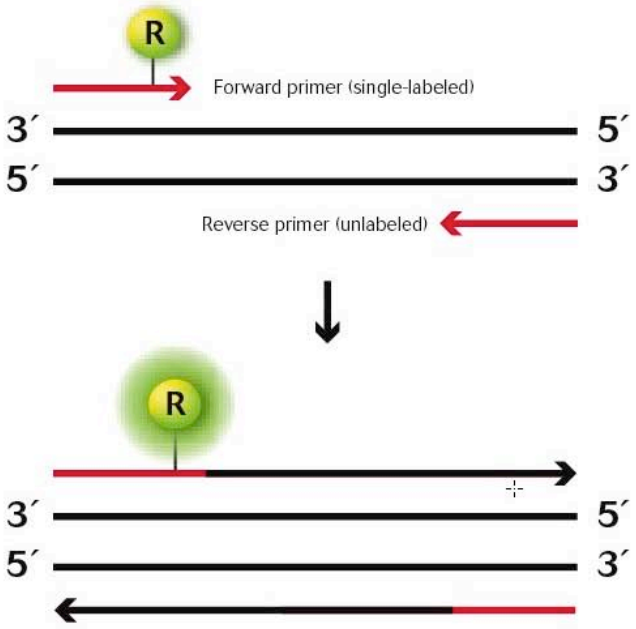
Primer dimer
(lower Tm than target)

Very powerful tool for troubleshooting and verification

TaqMan® and LUX™



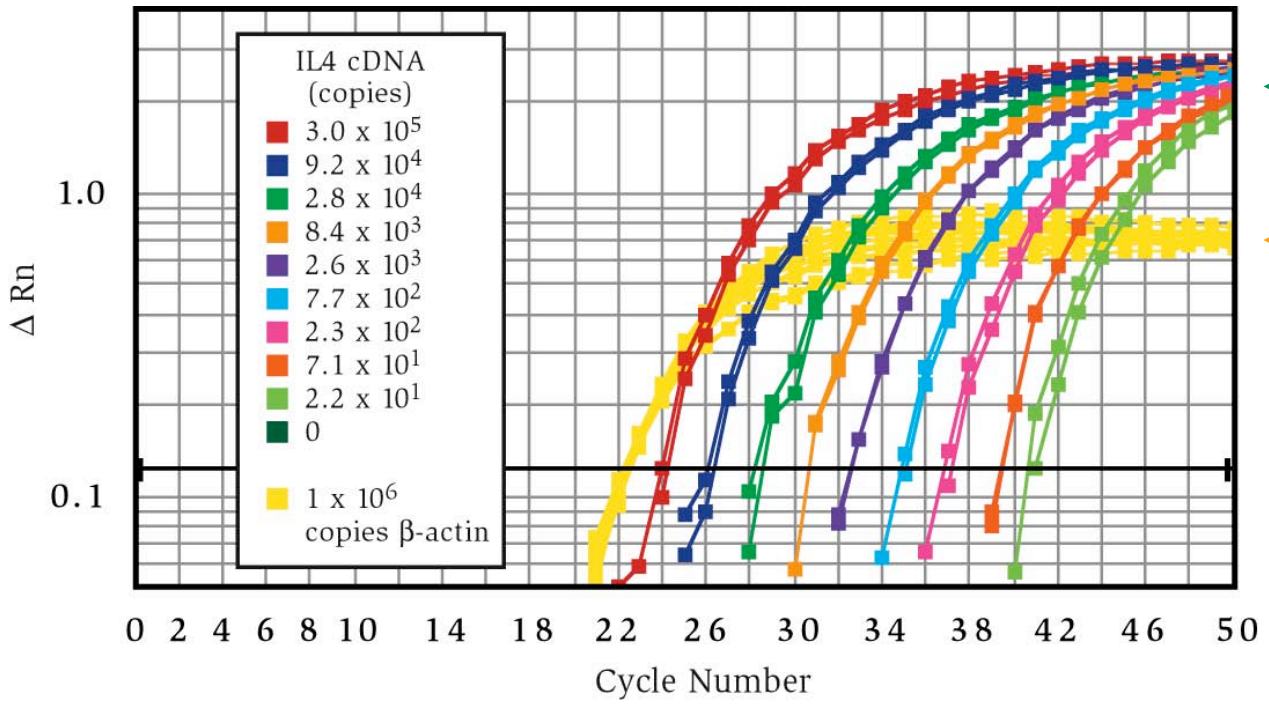
TaqMan



LUX

Both capable of multiplexing

Multiplex LUX™



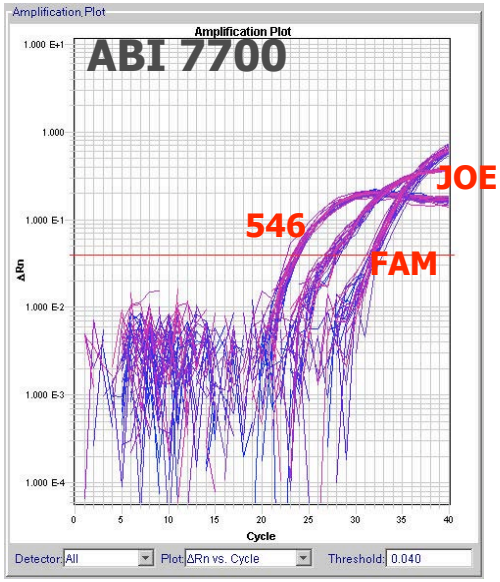
IL4 FAM (target)

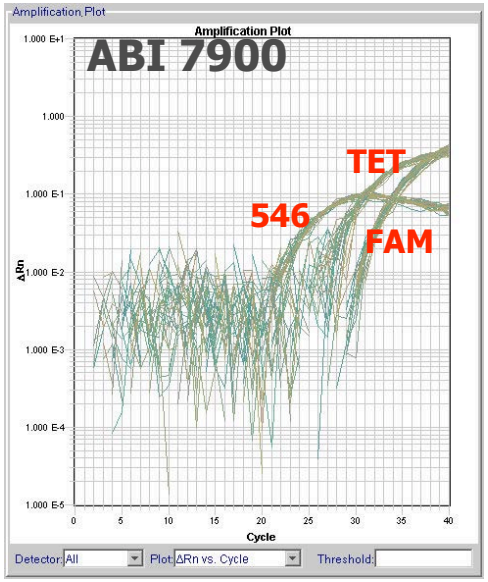
Beta Actin JOE
(internal reference)

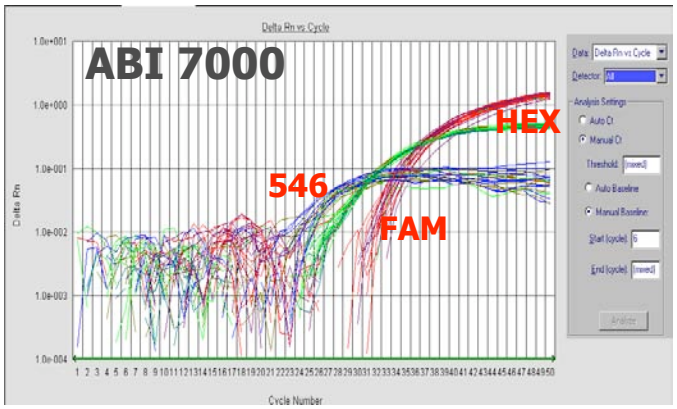
- 200nM each primer
- Platinum® Quantitative PCR SuperMix-UDG with ROX
- 50µl volumes

Triplex Reactions

Instrument	Recommended Dyes Duplex RXNs	Triplex RXNs	Comments
ABI 7000	FAM/JOE, FAM/HEX, FAM/TET, FAM/546	FAM/HEX/546	
ABI 7500	FAM/JOE, FAM/HEX, FAM/546	FAM/HEX/546, FAM/JOE/546	dilute ROX 1:10
ABI 7700	FAM/JOE, FAM/HEX, FAM/TET, FAM/546	FAM/JOE/546	
ABI 7900HT	FAM/JOE, FAM/HEX, FAM/TET, FAM/546	FAM/TET/546	
Rotor-gene	FAM/JOE, FAM/HEX, FAM/TET, FAM/546	N/A	







FAM = MAPK3 100 copies/rxn

JOE= PPIA 1,000 copies/rxn

ALEXA 546 = ActB 10,000 copies/rxn

Multiplexing Conditions

- Most LUX™ multiplex reactions run with Platinum® qPCR SuperMixes require no optimization
  - Mainly limited by instrument channels and dye
  - Up to 3-plex possible with most instruments

- If optimization is required
  1) decrease primer concentration for most abundant target
  2) increase $MgCl_2$ to 6mM
  3) add additional Platinum® *Taq*

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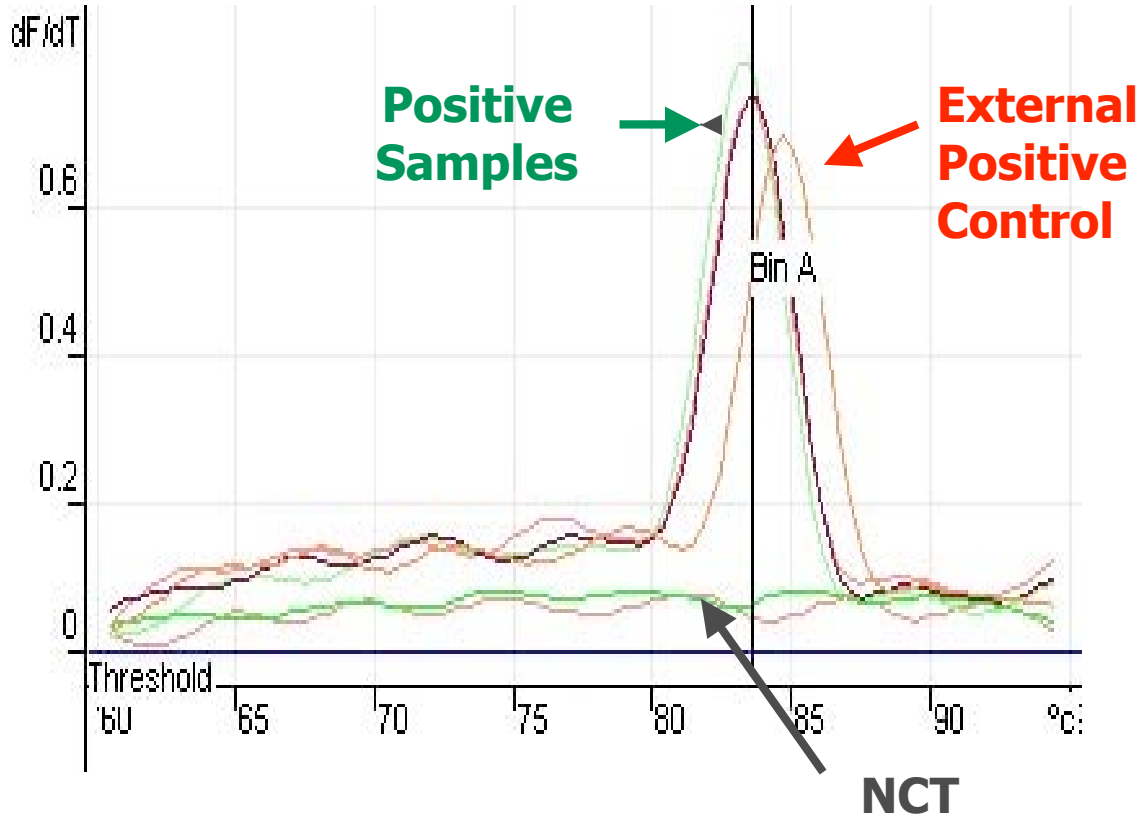
Comparison of Detection Chemistries

	TaqMan®	LUX™ primers	SYBR® Green
Sensitivity	+++	+++	++
Specificity	+++	+++	++
Dynamic range	+++	+++	++
Multiplexing	++	+++	-
Melting curve	-	+++	+++
Ease of primer/ probe design	+	+++	+++
Cost effectiveness	+	++	+++

The best of both worlds

External Positive Control

Anthracis
LUX™ Assay



Avoid false positives from EPC contamination

Data Submitted for Publication

Two-color Multiplex Assay for the Identification of Orthopox Viruses with Real-time LUX PCR

Aitichou, M.1, Javorschi, S.2 and Ibrahim, M.S.1
1. Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; and 2. Invitrogen, Corp., Carlsbad, CA

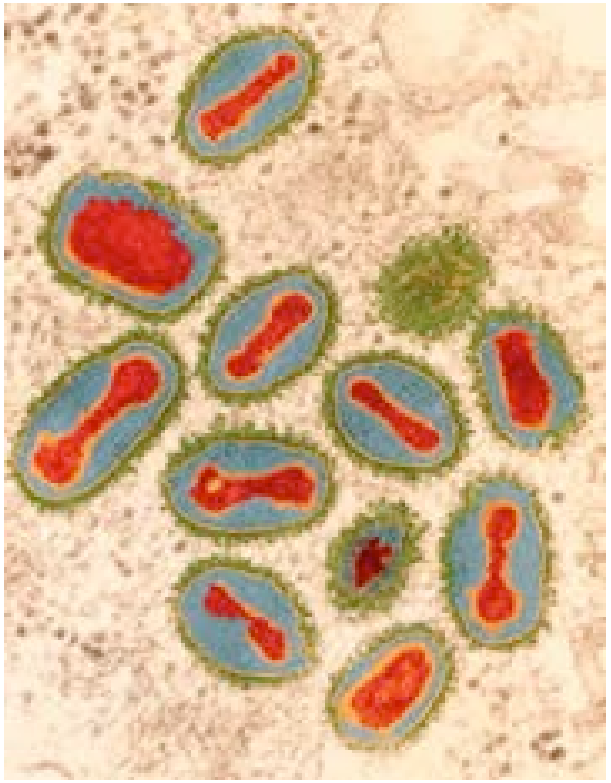
The LUX system (Light Upon eXtension) is a real-time detection platform that can be used for detecting and assaying pathogen nucleic acids. This system is PCR- based that uses one self-quenched fluorogenic primer labeled with a single fluorophore. The labeled primer emits fluorescence upon its incorporation and extension into the target nucleic acid sequence, and the fluorescence intensity is proportional to the amount of nucleic acids amplified during the PCR reaction. In this study, a highly sensitive and specific assay for identifying orthopox viruses was developed. The genomes of *Orthopoxvirus* species are extremely conserved and require a technology that can offer flexibility to enable high level of specificity. We used a variation of the LUX detection system, named Universal LUX platform. This technology enables the design of primer sets in the best area for detection specificity without following the design rules that apply to regular LUX primers. The assay is a real-time multiplex Universal LUX-PCR targeting the hemagglutinin gene sequence designed to allow simultaneous detection of *Variola* and other orthopox viruses. The detection limit of the assay was 50 and 100 copies for plasmid and genomic DNA, respectively, which represents 0.1 to 10 fg of DNA per reaction. These detection limits were highly reproducible. Regression analysis showed that the assay had linearity over seven logs with 0.97 correlation coefficient. The sensitivity and specificity were determined using a panel that consisted of 100 samples and controls. Both sensitivity and specificity were rated at 98%. Thus, the assay offers a sensitive, specific and quantitative tool for simultaneous detection of *Variola* and other orthopox viruses.

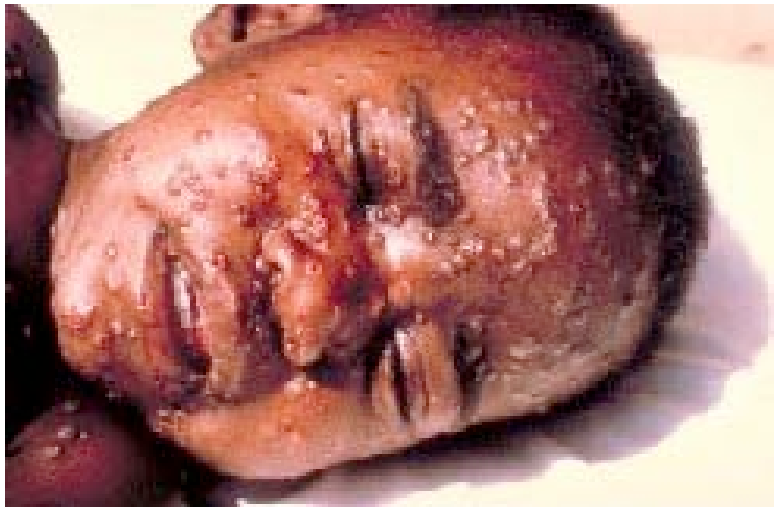
Background

- Orthopox viruses
  - Family *Poxviridae,* genus *Orthopoxvirus*
  - Double stranded DNA genome
- *Variola* is the agent of human smallpox
  - Emerged in human population thousands of years ago
  - World-wide elimination in 1980
  - Fatality rate is approximately 30% for unvaccinated persons
  - No specific therapies are available
    - Vaccination post-infection is usually effective

Background

- Vaccination
  - USA discontinued routine smallpox vaccination of children in 1972, of health care workers in 1976, and of Army recruits in 1990
  - Today, effectiveness of vaccinations performed in 60s and 70s is unknown
- *Vaccinia,* another Orthopox virus is used for immunization
  - >94% sequence homology with *Variola*





Objective

- To develop a system for rapidly identifying *Variola* and other Orthopox viruses
  - For example: has a soldier been exposed to smallpox, or simply vaccinated?

- Requirements
  - Sensitive
  - Cost effective
  - Multiplex capable
  - Melt curve to confirm positive result

Detection Method

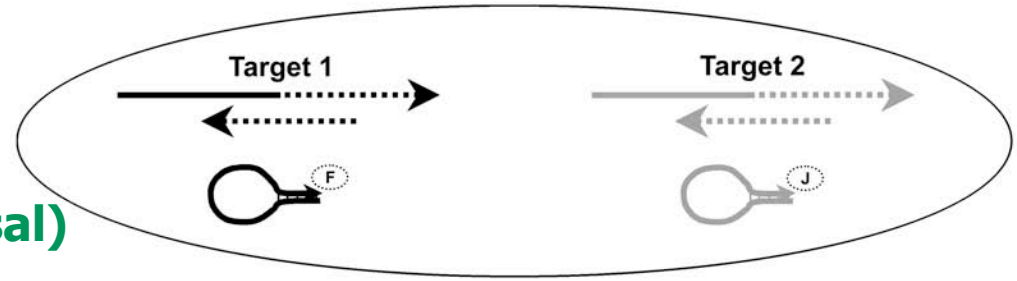
- Previously developed probe-based detection system has specificity issues due to high homology (>94%) between *Variola* and *Vaccinia*
  - Species-specific and species-wide assays required in same tube

- New approach proposed: Universal LUX™ detection system
  - Flexibility of primer design
  - Dissociation curve confirms positive result
    - Useful in typing non-*Variola* strains
  - Ease of multiplexing with good limit of detection
  - Very low assay cost

Universal LUX™ System

3 primers per target

(one is universal)



Provides additional design flexibility

Universal LUX™ for Orthopox Viruses

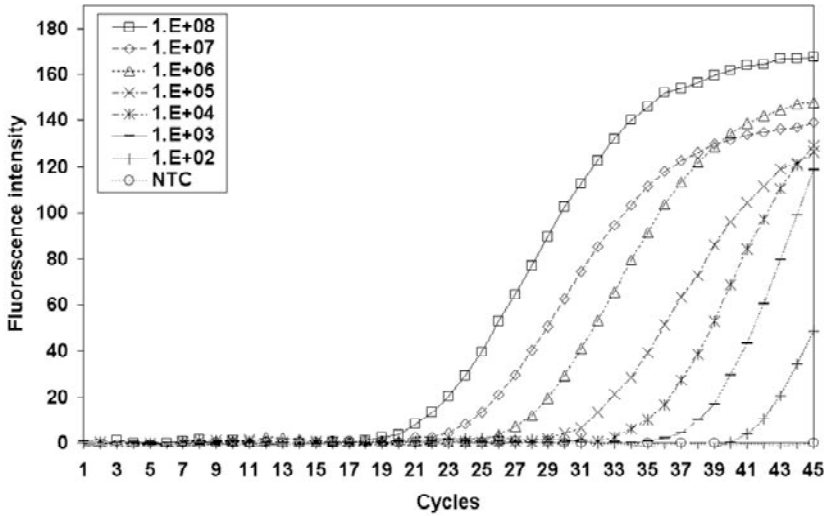
- 2 Universal LUX primer sets were designed:
  - Specific for Variola (universal JOE tail)
  - Specific for all Orthopox (universal FAM tail)

- NOTE: the PCR primers do not contain fluorescent labels. Each forward primer contains a 5' tag (UNIJ or UNIF) corresponding to a universal LUX primer labeled with FAM or JOE.

Uniform performance and low cost

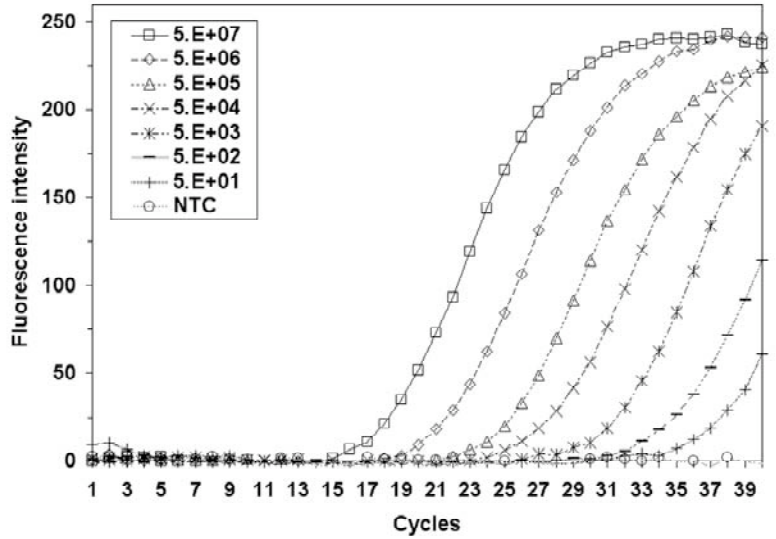
Results with Multiplex LUX™ Assays

Orthopox detection



Limit of detection of the multiplex assay expressed in copy numbers using monkeypox virus genomic DNA. Tenfold serial dilutions of monkeypox virus genomic DNA representing about 100 to 10,000,000 copies were tested. Each curve represents the mean fluorescence value of three replicates.

Variola detection



Limit of detection of the multiplex assay expressed in copy numbers using cloned *Variola* J7R DNA representing . Tenfold serial dilutions of *Variola* J7R DNA representing about 50 to 50,000,000 copies were tested. Each curve represent the mean fluorescence value of three replicates.

Good LOD--even in multiplex format

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Cross-reactivity Results

Species/sample	Strain/isolate	Conc. (fg)	*Variola* LUX	*Orthopoxvirus* LUX
Poxvirus samples				
Camelpox	Somalia	1000	0/2	2/2
Cowpox	Brighton	1000	0/2	2/2
Monkeypx	Zaire 1996-I-16	1000	0/2	2/2
Monkeypx	Zaire 1996-I-16	100	0/5	5/5
Monkeypx	Zaire 1996-I-16	10	0/6	5/6*
Myxoma	CDC	1000	0/2	0/2
Rabbitpox	CDC	1000	0/2	2/2
Racconpox	CDC	1000	0/2	2/2
Skunkpox	CDC	1000	0/2	0/2
Tanapox	CDC	1000	0/2	0/2
Vaccinia	BHS	1000	0/2	2/2
Vaccinia	CPN	1000	0/2	2/2
Variola J7R	BSH	10	6/6	6/6
Variola J7R	BSH	1	7/7	7/7
Variola J7R	BSH	0.1	12/12	12/12

Multiplex assay discriminated all strains as predicted

Conclusions

- We developed system for rapidly identifying and assaying *Variola* and other Orthopox viruses
  - Allows rapid confirmation or ruling out of a *Variola* infection in cases of accidental or deliberate exposure
  - Can be used as a tool to assess vaccine and anti-viral drug efficacy in animal studies
- Flexibility of design
- Ease of multiplexing
  - No optimization was required to convert from monoplex to duplex reactions
- Built-in control to prevent false positive and false negative results
- Low cost

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Acknowledgements

- This work was done in collaboration with Drs. Imrahim and Aitichou in the Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

- This research was supported by funding from the Defense Threat Reduction Agency, grant #02-4-4I-091 and by research program funds managed by the United States Army Medical Research and Materiel Command

LUX™ Product Portfolio

- **Certified LUX™ Primers**
  - Housekeeping Genes, human and mouse
  - 300+ Human Genes
  - Infectious Diseases
- **D-LUX™ Designer Software**
  - Design your own primers at www.invitrogen.com/lux
  - Enter sequence or accession number
  - Blast against public databases by species
  - Choose from 5 dye labels: FAM, JOE, HEX, TET, Alexa Fluor® 546