



Two-color multiplex assay for the identification of orthopox viruses with real-time LUX- PCR

M. Aitichou^{a,*}, S. Javorschi^b, M.S. Ibrahim^a

^a*Virology Division, United States Army Medical Research Institute of Infectious Diseases, SAMRIID, 1425 Porter St, Fort Detrick, MD 21702, USA*

^b*Invitrogen, Corp., Carlsbad, CA, USA*

Received 25 March 2005; accepted for publication 27 May 2005

Available online 14 July 2005

Abstract

The LUX[™] [Light Upon eXtension] is a real-time detection system that can be used for the detection and quantification of pathogens nucleic acids. In this study we used a universal LUX[™] approach, a variation of the LUX[™] detection system, for identifying *Orthopoxvirus* nucleic acids in real time. This approach enables the design of sequence-specific primer sets in high identity genome sequences. The assay described here is designed to allow simultaneous detection of *Variola* and other orthopox viruses in a multiplex format, with a limit of detection in the range of 50–100 copies of the *Orthopoxvirus* genome. Regression analysis showed that the assay was linear over seven orders of magnitude, with 0.97 correlation coefficient. The sensitivity and specificity of the assay, as determined from a panel of 100 samples that contained nucleic acids from a variety of bacteria and viral species, were rated at 98%. Thus, the assay offers a sensitive and specific tool for simultaneous identification and quantification of *Variola* and other orthopox viruses, and the approach allows more flexible sequence-specific primers design for pox viruses as well as other microbial pathogens.

© 2005 Elsevier Ltd. All rights reserved.

Keywords: Orthopoxvirus; LUX-PCR; Real-time PCR; Multiplex assay

1. Introduction

Members of genus *Orthopoxvirus* (Family *Poxviridae*) contain a double-stranded DNA genome that replicates in the cell cytoplasm. The most important member of this genus is the *Variola* virus, the agent of the now eradicated smallpox. Other orthopox viruses, such as monkeypox, cowpox, buffalopox and *Vaccinia* can also infect humans causing smallpox-like disease [1–10].

Several Real-time PCR methods for detecting orthopox viruses with high levels of sensitivity and specificity have been developed [11–18]. These reports used either SYBR green intercalating dye which has limited specificity or probes with a fluorophore and a quencher moieties, which could increase the cost of the assays.

The LUX[™] (Light Upon eXtension) is PCR-based detection system that uses one self-quenched fluorogenic

primer labeled with a single fluorophore. Typically 20–30 bases long, the oligonucleotide primer is designed with the fluorophore close to the 3' end in a hairpin structure. The fluorophore may be one of several commonly used dyes such as FAM [(5 or 6-carboxyfluorescein), TET (6-carboxy-4', 5'-dichloro-2',4,7,7'-tetrachlorofluorescein), JOE (6-carboxy-4,5-dichloro-2,7-dimethoxyfluorescein) or others. This configuration and the presence of a guanine or cytosine at the 3' end of the oligonucleotide intrinsically renders fluorescence quenching capability so that a separate quenching moiety is not needed. Upon incorporation and extension of the fluorescein-labeled primer during the PCR, the primer emits fluorescence whose intensity is proportional to the amount of amplified nucleic acids, and can be measured in real time by using a real time fluorescence detection instrument [19].

In this study, we used a universal LUX[™] approach, a variation of the LUX[™] detection system, for identifying *Orthopoxvirus* nucleic acids and distinguishing between the *Variola* virus and other orthopox viruses in real time in a single reaction tube. This approach enables the design of specific primers in sequences with high identity without following the design rules that apply to standard

* Corresponding author. Tel.: +1 301 619 2415; fax: +1 301 619 2290

E-mail address: mohamed.aitichou@det.amedd.army.mil (M. Aitichou).

Report Documentation Page				Form Approved OMB No. 0704-0188		
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.						
1. REPORT DATE 14 JUL 2005		2. REPORT TYPE N/A		3. DATES COVERED -		
4. TITLE AND SUBTITLE Two-color multiplex assay for the identification of orthopox viruses with real-time LUX-PCR, Molecular and Cellular Probes 19:323 - 328				5a. CONTRACT NUMBER		
				5b. GRANT NUMBER		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) AitIchou, MI Javorschi, S Ibrahim, MS				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD				8. PERFORMING ORGANIZATION REPORT NUMBER RPP-05-124		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)		
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited						
13. SUPPLEMENTARY NOTES The original document contains color images.						
14. ABSTRACT The LUXtrade mark [Light Upon eXtension] is a real-time detection system that can be used for the detection and quantification of pathogens nucleic acids. In this study we used a universal LUXtrade mark approach, a variation of the LUXtrade mark detection system, for identifying Orthopoxvirus nucleic acids in real time. This approach enables the design of sequence-specific primer sets in high identity genome sequences. The assay described here is designed to allow simultaneous detection of Variola and other orthopox viruses in a multiplex format, with a limit of detection in the range of 50-100 copies of the Orthopoxvirus genome. Regression analysis showed that the assay was linear over seven orders of magnitude, with 0.97 correlation coefficient. The sensitivity and specificity of the assay, as determined from a panel of 100 samples that contained nucleic acids from a variety of bacteria and viral species, were rated at 98%. Thus, the assay offers a sensitive and specific tool for simultaneous identification and quantification of Variola and other orthopox viruses, and the approach allows more flexible sequence-specific primers design for pox viruses as well as other microbial pathogens.						
15. SUBJECT TERMS methods, detection, LUX-PCR, orthopox viruses, sensitivity, specificity						
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT SAR	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified				

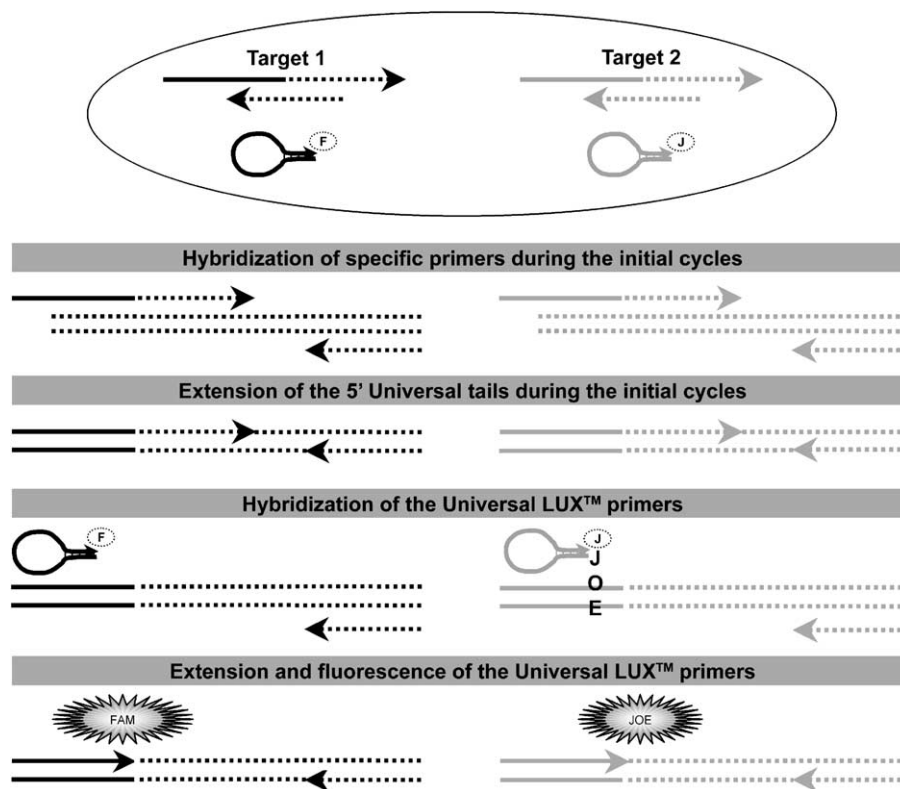


Fig. 1. Universal LUX™ in multiplex mode. A set of specific primers are designed for each of two targets. In each pair, one of the primers has a 5' end extension that has the same sequence as one of the 2 Universal LUX™ primers. In the initial cycles, only the specific primers are extended. After a few cycles, the Universal LUX™ primers are hybridized and produced Light Upon eXtension.

LUX™ primer design. This system consists of a labeled LUX™ primer with a unique sequence, designed not to hybridize with any sequences within known genomes. The Universal primer is coupled to gene-specific primers to amplify target sequences. The forward gene-specific primer is synthesized with a 5' extension identical to the sequence of the Universal LUX™ primer. During PCR, the extended primer anneals to the target sequence and is extended by DNA polymerase. The reverse unlabeled primer is then extended and the complementary sequence is synthesized, including the sequence complementary to the Universal LUX™ primer. In the later cycles, the Universal LUX™ primer anneals to this newly created sequence and is extended, generating a fluorescent signal (Fig. 1). Using two different Universal sequences, one labeled with a FAM dye and the second labeled with a JOE dye, this system can be used to design multiplex detection assays.

2. Materials and methods

2.1. Universal LUX™ primers

Two Universal LUX™ primers were designed with different fluorophores, FAM or JOE. Both primers were

designed to be roughly the same length, and have the same G+C content and melting temperature. Primers were blasted against public databases to confirm that they do not recognize sequences within unrelated genomes. The sequences of these Universal LUX™ primers are proprietary of Invitrogen, and are commercially available (Invitrogen, Carlsbad, CA).

2.2. Unlabeled primers

To amplify all orthopox virus DNA, we used ALL-FORWARD: 5'-TCATCTGGAGAATCCACAACA-3' and ALL-REVERSE: 5'-CAGTGTCTGTGACTGTATGATCTTC-3. To specifically amplify *Variola* DNA, we used VAR-FORWARD: 5'-AACAGACAAGACGTCGGG-3' and VAR-REVERSE: 5'-GTCTGTGACTGTATGATCTTTATT-3'. These specific primer sets were designed after analyzing all published hemagglutinin genes of orthopox viruses and using the criteria for standard real-time PCR primers design, including the primers length, T_m, GC contents, self-complementarity, amplicon size and minimizing primer-dimer formation. On each forward primer a 5' extension sequence corresponding to the proprietary sequences of the Universal LUX™ primer was added. All primers were synthesized by Invitrogen.

2.3. DNA samples

All viral and bacterial DNA samples used in this study were from the collections of the United States Army Medical Research Institute of Infectious Diseases and the Centers for Disease Control and Prevention, and are summarized in Table 1. DNAs included isolates of camelpox, cowpox, monkeypox, myxoma, tanapox, rabbitpox, raccoonpox, skunkpox, and *Vaccinia* viruses. The origins, propagation, and harvesting procedures for orthopox viruses are described in Ropp et al. (1995) [20]. DNA was extracted from virus-infected cells, virions, and scabs by using the Aquapure DNA kit (Bio-Rad). Prior experiments demonstrated that the material was noninfectious after 60 min of incubation at 55 °C in Aquapure lysis buffer.

2.4. RNA samples

Total RNA was extracted from virus-infected cell cultures by the TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. RNA pellets were dissolved in 5–10 µL of Rnase-free water with 40U of RnaseOUT recombinant inhibitor (Invitrogen) and stored at –80 °C until used.

2.5. Statistical analysis

Regression analysis was done by using Microsoft Excel to evaluate linearity and determine the quantitative performance of each assay.

2.6. Real-time quantitative PCR

The reactions were carried out in 30 µL volumes on the SmartCycler [Cepheid Inc., Sunnyvale, CA]. Each reaction consisted of 15 µL of Platinum[®] quantitative PCR Super-Mix—uracil DNA glycosylase (UDG). Final concentration of constituents was 20 mM Tris [pH 8.4]; 50 mM KCL; 0.2 µM of each dATP, dCTP, dGTP; 0.4 µM dUTP; 5 mM of MgCl₂; 0.45 U of Platinum Taq DNA polymerase; 0.3 U of UDG; stabilizers; and an additional 0.9 U of Platinum Taq DNA polymerase; 40 nM of each extended forward primers; 200 nM of each reverse primer; 200 nM of each Universal LUX[™] primer; additives [final concentration 0.2 mM Tris–HCL [pH 8.0], 0.2 mg/ml of bovine serum albumin, 150 mM of trehalose, 0.2% Tween 20], and 2 µL of template DNA.

PCR amplification was performed in a single tube on the SmartCycler as follows: one cycle of 50 °C for 2 min, one cycle of 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s and 60 °C for 45 s. The melting curve analysis was performed by denaturation at 95 °C for 10 s, followed by a step at 50 °C for 30 s. and heating to 80 °C with a rate of 0.1 °C/s, while monitoring the fluorescence in the temperature ramp. Fluorescence detection of *Orthopoxvirus* DNA

Table 1

Cross-reactivity results of the *Variola/Orthopoxvirus* multiplex assay

Species/sample	Strain/isolate	Conc. [fg]	<i>Variola</i> LUX	<i>Orthopox-virus</i> LUX
Poxvirus samples				
Camelpox	Somalia	1000	0/2	2/2
Cowpox	Brighton	1000	0/2	2/2
Monkeypox	Zaire 1996-I-16	1000	0/2	2/2
Monkeypox	Zaire 1996-I-16	100	0/5	5/5
Monkeypox	Zaire 1996-I-16	10	0/6	5/6 ^a
Myxoma	CDC	1000	0/2	0/2
Rabbitpox	CDC	1000	0/2	2/2
Raccoonpox	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
Bacterial samples				
<i>Bacillus anthracis</i>	4728	1000	0/2	0/2
<i>Clostridium perfringens</i>	13124	1000	0/2	0/2
<i>Francisella tularensis</i>	NA	1000	0/2	0/2
<i>Haemophilus influenzae</i>	10211	1000	0/2	0/2
<i>Listeria monocytogenes</i>	15313	1000	0/2	0/2
<i>Neisseria lactamica</i>	23970	1000	0/2	0/2
<i>Staphylococcus aureus</i>	25923	1000	0/2	0/2
<i>Staphylococcus hominis</i>	27844	1000	0/2	0/2
<i>Streptococcus pyogenes</i>	19615	1000	0/2	0/2
<i>Yersinia pseudotuberculosis</i>	690	1000	0/2	0/2
Viral samples				
Black Creek Canal	39179	100	0/2	0/2
Dengue 3	CH3489	100	0/2	0/2
Eastern Equine Encephalitis	FL-4679	100	0/2	0/2
Ebola	Boniface	100	0/2	0/2
Ebola	Zaire95	100	0/2	0/2
Hantaan	76-118	100	0/2	0/2
Lassa	Josiah	100	0/2	0/2
Rift Valley fever	ZH 548	100	0/2	0/2
Venezuelan Equine Encephalitis	PE-4.0904	100	0/2	0/2
West Nile	Crow 397-99	100	0/2	0/2
Yellow fever	Assibe	100	0/2	1/2 ^b
Other samples				
Human genomic DNA	NA	1000	0/2	0/2
Total			100	100

NA not applicable. One hundred samples were randomly tested; 25/25 *Variola* DNA samples were correctly detected; 24/25 genomic DNA from orthopox viruses were detected; 49/50 negative samples were not detected. Therefore, the sensitivity and specificity were rated at 98%.

^a False negative.

^b False positive.

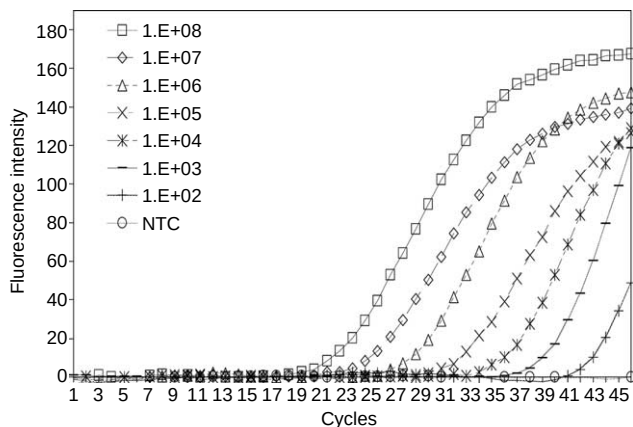


Fig. 2. Limit of detection of the multiplex assay expressed in copy numbers using monkeypox virus genomic DNA. Tenfold serial dilutions of monkeypox virus genomic DNA representing about 100–100,000,000 copies were tested. Each curve represents the mean fluorescence value of three replicates.

was monitored on the SmartCycler by using the FAM channel and detection of *Variola* DNA by using the TET channel.

3. Results

Assay performance characteristics were evaluated by determining the lower concentration of DNA that could be detected, the linearity of a range of different DNA concentrations and the ability of the assay to specifically identify *Variola* virus and other orthopox viruses among a panel of unrelated bacterial and viral nucleic acids samples.

To determine the assay limit of detection (LOD), serial 10-fold dilutions from *Variola* J7R cloned DNA [13] and monkeypox virus DNA representing 50–100,000,000 copies of DNA were analyzed by the two-color assay in two separate runs. The LOD of *Variola* virus J7R cloned DNA

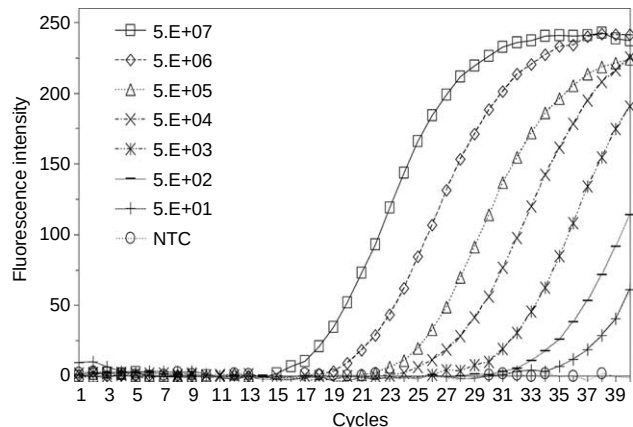


Fig. 3. Limit of detection of the multiplex assay expressed in copy numbers using cloned *Variola* J7R DNA. Tenfold serial dilutions of *Variola* J7R DNA representing about 50 to 50,000,000 copies were tested. Each curve represents the mean fluorescence value of three replicates.

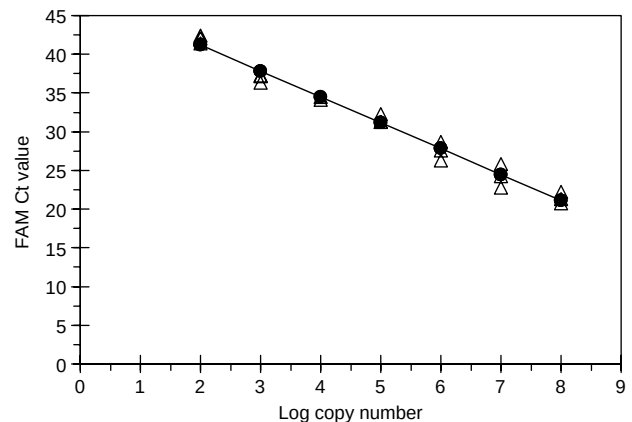


Fig. 4. Regression analysis on the C_t value against log copy numbers showing the dynamic range of the multiplex assays with monkeypox virus DNA preparations ranging from 100 to 100,000,000 copies [$R^2=0.98$; significance $F=2.8 \times 10^{-17}$].

was approximately 50 copies (Fig. 2) and that of monkeypox virus genomic DNA was 100 copies (Fig. 3). Regression analyses showed a strong linear correlation ($R^2=0.97$) between the C_t values and DNA concentrations over seven orders of magnitude for both FAM and JOE reporters of the multiplex assay (Fig. 4, Fig. 5).

The LOD of each multiplex assay was tested for reproducibility. Of 60 test repetitions with 50 copies of the *Variola* virus J7R plasmid DNA, 59 were detected with a mean C_t value of 35.07 ± 1.86 , and 58 of 60 test repetitions with 100 copies of monkeypox virus genomic DNA were detected with a mean C_t value of 34.9 ± 1.76 (Fig. 6).

The results of the sensitivity and specificity tests of the two-color assay are shown in Table 1. The *Variola* virus probe detected 25 of 25 samples containing *Variola* J7R DNA. The *Orthopoxvirus* probe detected 24 of the 25 DNA samples containing camelpox, cowpox, racconpox, *Vaccinia*, and monkeypox viruses. One false negative was obtained from samples of monkeypox DNA. Of the 50 negative samples, one

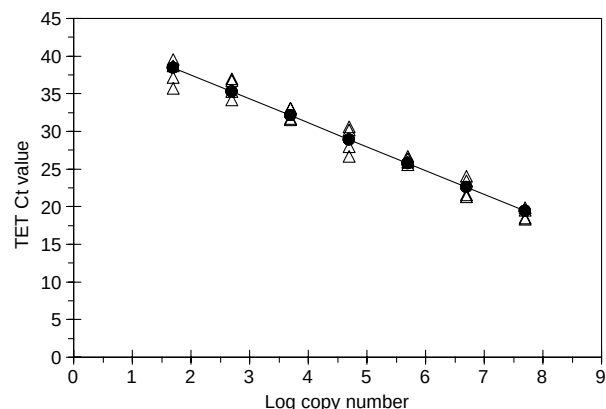


Fig. 5. Regression analysis on the C_t value against log copy numbers showing the dynamic range of the multiplex assay with cloned *Variola* J7R DNA preparations ranging from 50 to 50,000,000 copies [$R^2=0.97$; significance $F=8 \times 10^{-21}$].

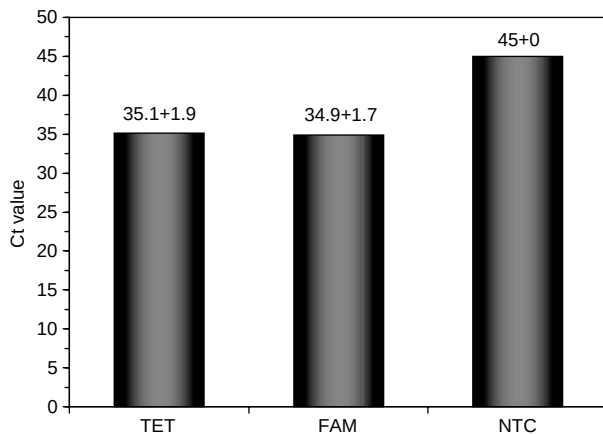


Fig. 6. Reproducibility of multiplex assays was expressed as the average C_t values of 60 repetitions. The mean C_t value and standard deviation are indicated on top of each bar.

was a false positive. Therefore, both sensitivity and specificity of this two-color multiplex assay were rated at 98%. It is worth mentioning that the false positive was obtained from a yellow fever virus samples that was unrelated to orthopox virus, suggesting that the false positive results were probably due to mishandling of the DNA samples. When the false positive and false negative samples were re-tested, both samples were correctly identified.

4. Discussion

The genomes of *Orthopoxvirus* species are extremely conserved and require a technology that can offer flexibility in primers design to enable high level of specificity for species differentiation. We developed a multiplex Universal LUXTM PCR assay for simultaneous detection and differentiation of *Variola* virus from other orthopox viruses.

Several publications have reported the use of SYBR green or dual-labeled probes for detecting orthopox viruses [11–16]. As of today, there have been no reports of single-labeled probe usage, such as in the LUXTM system, for orthopox virus diagnosis. LUXTM-PCR has several advantages over SYBR green or dual-labeled probes real-time PCR. It is a cost-effective assay, more amenable to multiplexing, and allows robust melting curve analysis of PCR product. This is possible because PCR products retain the fluorescent label after extension of the LUXTM primers. The melting curve can be used to resolve problems of primer dimers and false results. Primer dimers usually have a lower melting point (75 °C) than those of PCR products (78–90 °C). Reported real-time PCR methods that collect data in the exponential growth phase such as the fluorogenic 5' exonuclease and minor groove binding (MGB) probe-based PCR do not perform melting curve analysis. Besides the LUXTM system, the only cheaper alternative for melting curve analysis is the double-stranded DNA binding dye chemistry, such as SYBR-green. However, with this

chemistry, unspecific fluorescence intercalation occurs due to non-specific amplification and primer-dimer formation. Another disadvantage of the SYBR green-based real-time PCR chemistry is the requirement for extensive optimization [21]. Other published assays that used melting curve analysis included two probes, anchor and sensor probes, and dual-labelled probes [17,18].

Our multiplex assay had a good sensitivity level and linearity with a broad dynamic range. The assay detected at least 50 copies of the *Variola* virus cloned DNA (0.1 fg) and 100 copies of monkeypox virus genomic DNA (10 fg). With this assay, it was also possible to detect less than 10 fg of DNA with 75% reproducibility rate (data not shown). However, we elected to present an LOD (10 fg) because it was highly reproducible at a rate of at least 96% (58/60 repetitions). Previous studies using non-multiplexed Real-time PCR reported LOD between 4 and 250 copies [13,15–18]. It is noteworthy that the variations in LOD among different assays depend on a number of factors, including the target sequence, the assay platform, and more importantly the purity of the DNA template. In our study, the concentration of genomic DNA used to determine LODs was determined by a spectrophotometer with an absorbance 260/280 ratio of 1.75. Although, our assay is a multiplex assay, its LOD remains within the range of LODs of non-multiplexed assays reported previously and is well below the range of viral load expected in clinical samples. For example, up to 10^6 pock-forming units or 200 pg of *Variola* DNA were obtained from skin lesions of the smallpox rash by day 2 of the rash [18,22].

The performance of the LUXTM assay was evaluated in terms of clinical sensitivity and specificity by testing 100 DNA samples that were obtained from a variety of bacterial and viral species. The assay was highly sensitive (98%) and specific (98%), indicating its utility for clinical samples testing, particularly because of its ability to distinguish between *Variola* and other *Orthopoxvirus* infections in a single tube reaction. Only one false negative and one false positive result were obtained. These two samples were unambiguously clarified first, by melting curve analysis and second by re-testing the samples three times to confirm the results.

In conclusion, the assay presented here should be helpful for rapidly identifying and assaying *Variola* and other orthopox viruses, allowing the opportunity to confirm or rule out a *Variola* infection should such an infection occur accidentally or deliberately. Furthermore, the assay can be used as a quantitative tool to assess viral loads in vaccine and anti-viral drug efficacy studies.

Acknowledgements

This work was supported by research program funds managed by the United States Army Medical Research and Materiel Command, Fort Detrick, MD. We thank Kathryn Kenyon for reviewing the manuscript. The mention of materials or products in this article does not constitute

endorsement by the Department of Defense or the United States government. This research was supported by funding from the Defense Threat Reduction Agency, grant #02-4-4I-091.

References

- [1] Hutin YJ, Williams RJ, Malfait P, Pebody R, Loparev VN, Ropp SL, et al. Outbreak of human monkeypox, Democratic Republic of Congo, 1996 to 1997. *Emerg Infect Dis* 2001;7:434–8.
- [2] Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, Wegner M V, et al. The detection of monkeypox in humans in the Western Hemisphere. *N Engl J Med* 2004;350:342–50.
- [3] Stephenson J. Monkeypox outbreak a reminder of emerging infections vulnerabilities. *JAMA* 2003;290:23–4.
- [4] Ligon BL. Monkeypox: a review of the history and emergence in the Western hemisphere. *Semin Pediatr Infect Dis* 2004;15:280–7.
- [5] Egan C, Kelly CD, Rush-Wilson K, Davis SW, Samsonoff WA, Pfeiffer H, et al. Laboratory-confirmed transmission of Vaccinia virus infection through sexual contact with a military vaccinee. *J Clin Microbiol* 2004;42:5409–11.
- [6] Hawranek T, Tritscher M, Muss WH, Jecel J, Nowotny N, Kolodziejek J, et al. Feline orthopox virus infection transmitted from cat to human. *J Am Acad Dermatol* 2003;49:513–8.
- [7] Heilbronner C, Harzic M, Ferchal F, Pothier A, Charara O, Beal G, et al. Cowpox virus infection in a child. *Arch Pediatr* 2004;11:335–9.
- [8] Meyer H, Perrichot M, Stemmler M, Emmerich P, Schmitz H, Varaine F, et al. Outbreaks of disease suspected of being due to human monkeypox virus infection in the Democratic Republic of Congo in 2001. *J Clin Microbiol* 2002;40:2919–21.
- [9] Jezek Z, Arita I, Mutombo M, Dunn C, Nakano JH, Szczeniowski M. Four generations of probable person-to-person transmission of human monkeypox. *Am J Epidemiol* 1986;123:1004–12.
- [10] Edmiston Jr CE, Graham MB, Wilson PJ, Grahn B. The monkeypox virus outbreak: reflections from the frontlines. *Am J Infect Contr* 2003;31:382–4.
- [11] Ibrahim MS, Esposito JJ, Jahrling PB, Lofts RS. The potential of 5' nuclease PCR for detecting a single base polymorphism in orthopoxvirus. *Mol Cell Probes* 1997;11:143–7.
- [12] Ibrahim MS, Lofts RS, Jahrling PB, Henchal EA, Weedn VW, Northrup MA, et al. Real-time microchip PCR for detecting single-base differences in viral and human DNA. *Anal Chem* 1998;70:2013–7.
- [13] Ibrahim MS, Kulesh DA, Saleh SS, Damon IK, Esposito JJ, Schmaljohn AL, et al. Real-time PCR assay to detect smallpox virus. *J Clin Microbiol* 2003;41:3835–9.
- [14] Espy MJ, Cockerill III FR, Meyer RF, Poland MD, Hadfield TL, Smith TF. Detection of smallpox virus DNA by LightCycler PCR. *J Clin Microbiol* 2002;40:1985–8.
- [15] Kulesh DA, Baker RO, Loveless BM, Norwood D, Zwiers SH, Mucker E, Hartmann C, et al. Smallpox and pan-orthopox virus detection by real-time 3'-minor groove binder TaqMan assays on the Roche lightCycler and the cepheid smartcycler platforms. *J Clin Microbiol* 2004;42:601–9.
- [16] Kulesh DA, Loveless BM, Norwood D, Garrison J, Whitehouse CA, Hartmann C, Mucker E, et al. Monkeypox virus detection in rodents using real-time 3'-minor groove binder TaqMan assays on the Roche LightCycler. *Lab Invest* 2004;84:1200–8.
- [17] Nitsche A, Ellerbrok H, Pauli G. Detection of orthopoxvirus DNA by real-time PCR and identification of variola virus DNA by melting analysis. *J Clin Microbiol* 2004;42:1207–13.
- [18] Olson VA, Laue T, Laker MT, Babkin IV, Drosten C, Shchelkunov SN, et al. system for detection of orthopoxviruses and simultaneous identification of smallpox virus. *J Clin Microbiol* 2004;42:1940–6.
- [19] Nazarenko I, Lowe B, Darfler M, Ikononi P, Schuster D, Rashtchian A. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res* 2002;30:e37.
- [20] Ropp SL, Jin Q, Knight JC, Massung RF, Esposito JJ. PCR strategy for identification and differentiation of small pox and other orthopoxviruses. *J Clin Microbiol* 1995;33:2069–76.
- [21] Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *Biotechniques* 1998;24:954–8 see also pages 960 and 962.
- [22] Damon IK, Esposito JJ. Poxvirus infections. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH, editors. *Manual of clinical microbiology*. 8th ed. Washington, DC: American Society for Microbiology Press; 2003. p. 1583–91.