

Development of a Real-Time Reverse Transcription PCR Multiplex Assay to Screen for Eastern, Western and Venezuelan Equine Encephalitis

Michael Powers¹, Binxue Zhang², Dongxiang Xia², Randy Rasmussen¹, Deepika de Silva¹, Ted L Hadfield²

¹Idaho Technology Inc., Salt Lake City, Utah, ²Armed Forces Institute of Pathology, Washington DC

Contact Information
mikep@idahotech.com
deepika@idahotech.com
hadfield@afip.osd.mil

C-318

Summary

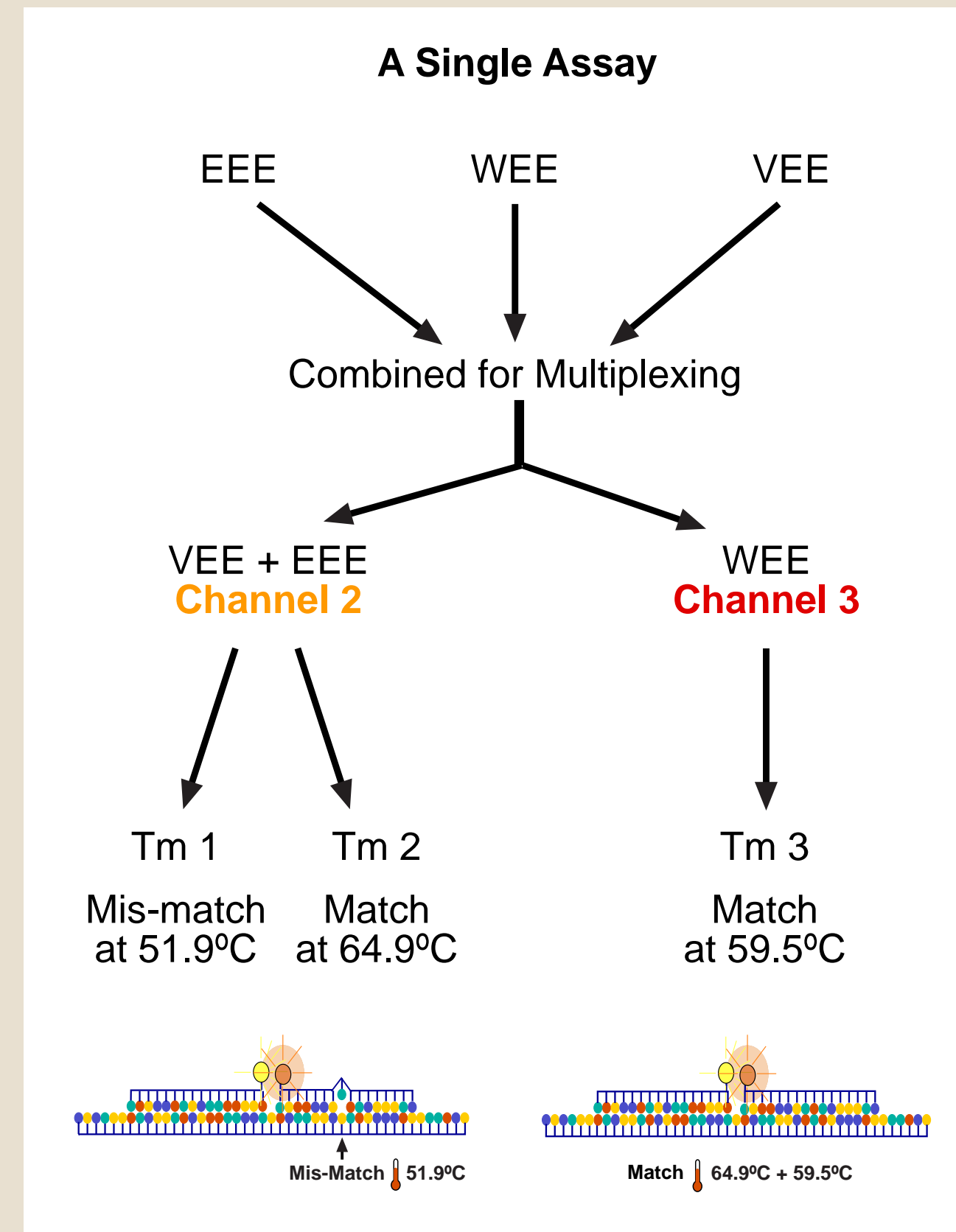
Development of a real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) multiplex assay, allowing for the screening of Eastern, Western and Venezuelan Equine Encephalitis pathogens in a single capillary.

Abstract

The US Department of Defense has classified multiple viral agents as potential biothreat agents suitable for bio-terrorism. Agents such as smallpox, viral encephalitis and others are of concern due to their infectious nature. The risk is magnified due to the fact that large populations are susceptible to these agents, while only limited treatment and vaccination strategies exist. It is important that public health organizations and health care providers have a means of screening the site and people at the location of an outbreak in the event of such a bio-terrorist act. With this in mind, we have developed a real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) multiplex assay, utilizing hybridization probes. This assay allows for the screening of Eastern, Western and Venezuelan Equine Encephalitis pathogens in a single capillary on the Idaho Technology Ruggedized Advanced Pathogen Identification Device known as the R.A.P.I.D.[®] System. The multiplexed assay is designed to detect two of the three pathogens in a single channel using the dye LC-Red 640. These two targets are distinguished by melting temperature analysis (T_m), while the third pathogen is detected in a different channel by the use of a second dye (LC-Red 705). **Results:** The multiplex assay is robust with a sensitivity of 5pg/rxn of total RNA. We observed a limit of detection for Eastern and Venezuelan Equine Encephalitis of 5pg/μl of total RNA. While the limit of detection for Western Equine Encephalitis was 50pg/μl of total RNA. The experiments to determine sensitivity of this multiplex assay based upon viral PFU are ongoing. **Conclusion:** The use of this multiplex assay will allow for the simultaneous screening of three viral encephalitis pathogens, reducing time and cost of surveillance.

Introduction

Eastern, Venezuelan and Western Equine Encephalitis pathogens are zoonotic viruses of the genus *Alphavirus*, in the family *Togaviridae*, which are transmitted to equines and humans by mosquitoes. This virus places residents and visitors of endemic areas at risk. Symptoms in an infected individual range from mild flu-like to advanced encephalitis, coma and death. Initially two unique RT-PCR assays for the three encephalitis viruses EEE (E1, E2), WEE (E1, E2) and VEE (Nsp4, E1) were designed. These single-target assays were used in combination as a basis for the multiplex assay. These initial assays were designed to run under identical conditions with similar melting temperatures and were detected in the channel at an emission of 640nm. This allowed any combination of these assays to be run simultaneously on a single R.A.P.I.D. as either a Batch test (many gene targets within the same pathogen) or a Screen test (a sample screened for many pathogens). In order to perform a multiplex reaction, however, some of these assays required modification. When unmodified, the products of the two targets detected using LC-Red 640 (Channel 2) could not be distinguished based upon the probe melting temperature. A melting temperature difference of more than 6°C is required to observe baseline resolution between the two products after the melting analysis is completed. The use of melting temperature to distinguish products in the same detection channel is called temperature multiplexing (T_m multiplexing). The single assays were reviewed based upon probe melting temperature (T_m) and cross-complementarity to determine which assays would be used in the multiplex reaction.



Material and Methods

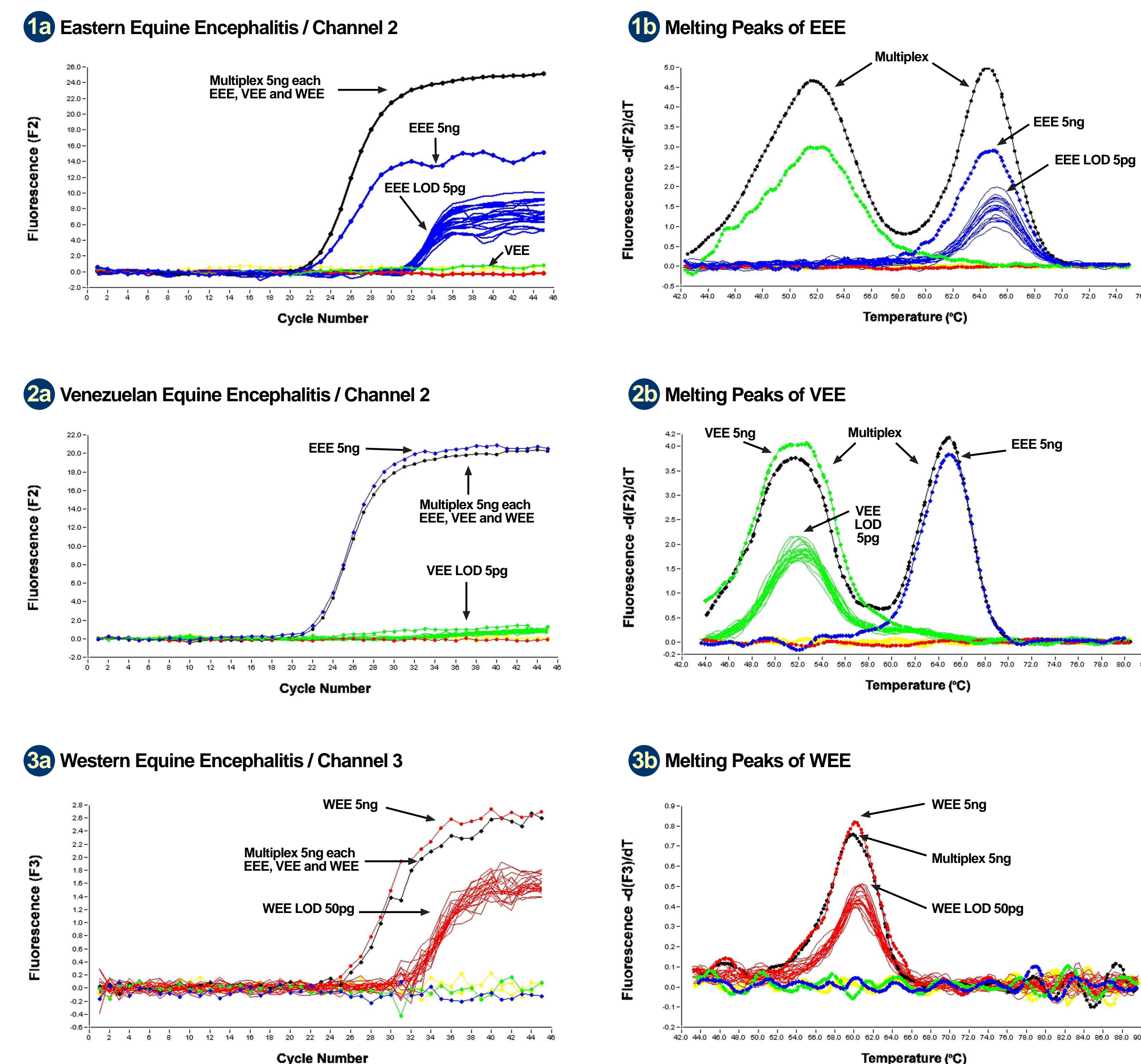
Nucleic Acid Preparation - Samples of culture supernatant from viral infected monolayer cultures were collected and spun at 2000 rpm for 10 minutes at 4°C. The supernatant was removed and filtered using a 0.45 mm filter. 200 units of Rnase-free Dnase was added to 10 ml of supernatant and incubated at 37°C for 30 minutes. 20 ml of Proteinase K (20 mg/ml) and 1 ml of 10% SDS were added to the supernatant and incubated at 55°C for 30 minutes. The sample was vortexed twice during the incubation period. Following the incubation, the sample was extracted twice with phenol/chloroform/isoamylalcohol (25:24:1) and once with chloroform/isoamylalcohol (24:1). The nucleic acids were precipitated with 3 volumes of ethanol and 1/10 volume of 3 M NaAc on ice for 30 minutes. The sample was precipitated by centrifugation at 12,000 rpm for 15 minutes. The pellet was washed once with 80% ethanol and air-dried. The RNA was dissolved in 100 μl TE buffer (pH 8.0). All samples were stored at -20°C.

Primers and Probes - Primers and probes were designed to identify EEE, VEE and WEE. To assure specificity of the primers, an alignment of similar genes from related organisms was performed using GenBanks Basic Local Alignment Search Tool (BLAST[®]). Two primer and probe sets for each EEE, VEE and WEE were designed using the Roche LightCycler[®] Primer Probe[™] software. The size of each gene target is between 125 and 194 base pairs. Detection probes were labeled with LC-Red 640 (Channel 2) and LC-Red 705 (Channel 3) fluorescence dyes.

Reverse Transcription PCR (RT-PCR) Conditions - Real-time RT-PCR analysis was performed using the R.A.P.I.D. System. RT-PCR reactions were performed in capillary tubes in 20 μL volumes. Each capillary contains all of the primers and probes to detect all three viral targets. Buffer contains MgCl₂ buffer, dNTPs, DTT, RNaseOUT, M-MuLV (Roche), KlenTaq1 DNA polymerase, TaqStart antibody, and enzyme diluent.

Eastern Equine Encephalitis Envelope Glycoprotein E2, Ch. 2
Venezuelan Equine Encephalitis Non-Structural Protein 4, Ch. 2
Western Equine Encephalitis Envelope Glycoprotein E2, Ch. 3
Multiplex EEE, VEE and WEE
No Template Control

Observed Melting Temperatures
EEE Channel 2 at 64.9°C
VEE Channel 2 at 51.9°C
WEE Channel 3 at 59.5°C



Results

The single assays for EEE (E2), VEE (Nsp4) and WEE (E2) were placed together to function in a multiplex fashion in a single capillary. As mentioned earlier, a mis-match was placed into the VEE detection probe sequence and the dye of the detection probe for WEE was changed to LC-Red 705. These modifications resulted in a multiplex assay that is capable of producing limits of detection of 5pg/rxn of total viral RNA for both EEE and VEE and 50pg/rxn of WEE (Table 1).

Table 1. LOD and Sensitivity reported in pg of total RNA/rxn

	EEE	VEE	WEE
LOD	5	5	50
Sensitivity	0.5	5	50

Discussion

Design of a multiplex PCR reaction requires the selection of primers and probes containing the lowest amount of cross-complementarity or primer dimer formation as possible. Once the oligo sequences have been determined, the melting temperatures (T_m) of the probes must be adjusted. This adjustment of T_m allows for two gene targets to be distinguished in one detection channel. Melting temperature of the probes can be adjusted by length of the probe or by the addition of a mis-match placed in the detection probe, as in this case. In this three-target multiplex reaction, EEE and VEE are detected in channel 2 using LC-Red 640 after the melting analysis, while WEE is detected in Channel 3. Some modifications were made to produce a functional multiplex assay. The assay for EEE (E2) was left unmodified; the assay for WEE (E2) was changed so the detection probe was labeled with LC-Red 705; the assay for VEE (Nsp4) was modified to contain a mis-match (G to A) in the middle of the detection probe sequence (LC-Red 640). This lowers the signature T_m of the probe used to identify the product, resulting in a new T_m of 52°C. The gene targets of EEE and VEE are detected in Channel 2 using the fluorescent dye LC-Red 640. Due to the placement of both of these gene targets in the same detection channel, the quantification is not informative as these two products cannot be simultaneously resolved during amplification. However, upon reviewing the melting data from these two gene targets, melting peaks of 52°C (VEE) and 64.9°C (EEE) are observed. Color compensation is required when using a R.A.P.I.D. or a LightCycler for accurate analysis. While each of these dyes has different emission maxima, and the detection filters have been optimized to these maxima, there is some spillover between channels. This spillover can be corrected by color compensation. The results reported represent only a limited number of strains available from AFIP. To date, an Alphavirus panel to test for specificity and cross-reactivity of these assays is unavailable.

Conclusion

Multiplexing is an effective methodology to screen large numbers of unknown samples in a cost-effective and timely fashion. Using this multiplex assay allows for the simultaneous screening of three viral encephalitis pathogens, reducing time and cost of surveillance. When the time and cost of surveillance is reduced, a wider range of surveillance is possible.

Acknowledgment

Funded in part by Grant DAMD 17-01-P-0447 USAMRIID. The authors wish to thank Mark Kessler for his graphical assistance and Rebecca Bowes for her editing assistance.