

Development of a Real-Time Reverse Transcription PCR Multiplex Assay for the Detection of Eastern Equine Encephalitis

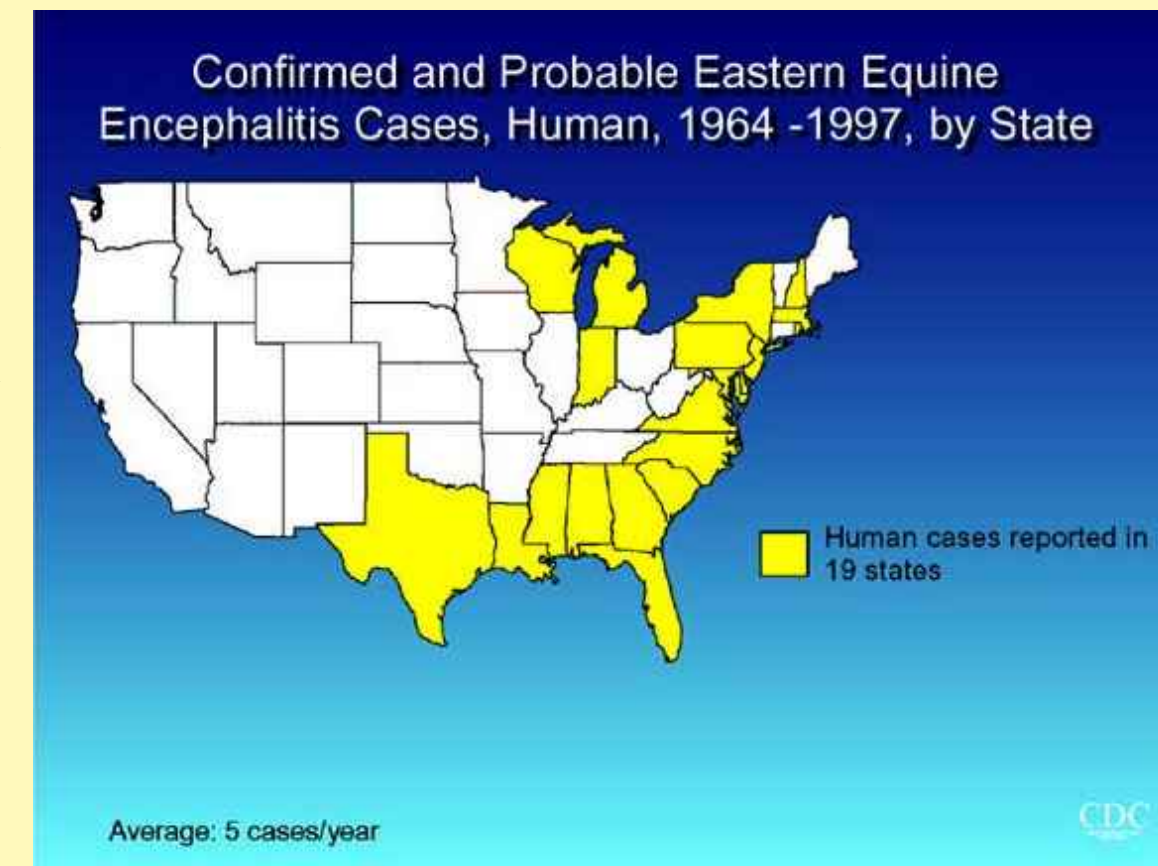
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Abstract

Background: Eastern Equine Encephalitis (EEE) is a mosquito-borne virus that has been classified by the US Department of Defense as a threat agent because of its potential use in bio-terrorism. The incidence of EEE infection in humans is low with only 153 cases reported since 1964. Yet, the cost of treating an infected individual ranges from \$21,000 to \$3 million. Population intrusion into endemic areas will create a need for increased surveillance and mosquito control programs.



Methods: We have developed a real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) multiplex assay in a single tube for the surveillance of EEE virus. This assay was designed to run on the Idaho Technology Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.®) System. We utilized a paired hybridization probe strategy. One oligo nucleotide probe contains a 3' label of fluorescein isothiocyanate (FITC) and the second probe is labeled 5' with either LC-Red 640 or LC-Red 705. When the probes are hybridized to the amplicon, the two dyes are brought into close proximity allowing energy transfer. The multiplex assay detects two gene targets. Target one, Envelope glycoprotein E1, is detected in channel 2 utilizing the dye LC-Red 640. Envelope glycoprotein E2, the second target, is detected in channel 3 utilizing the dye LC-Red 705.

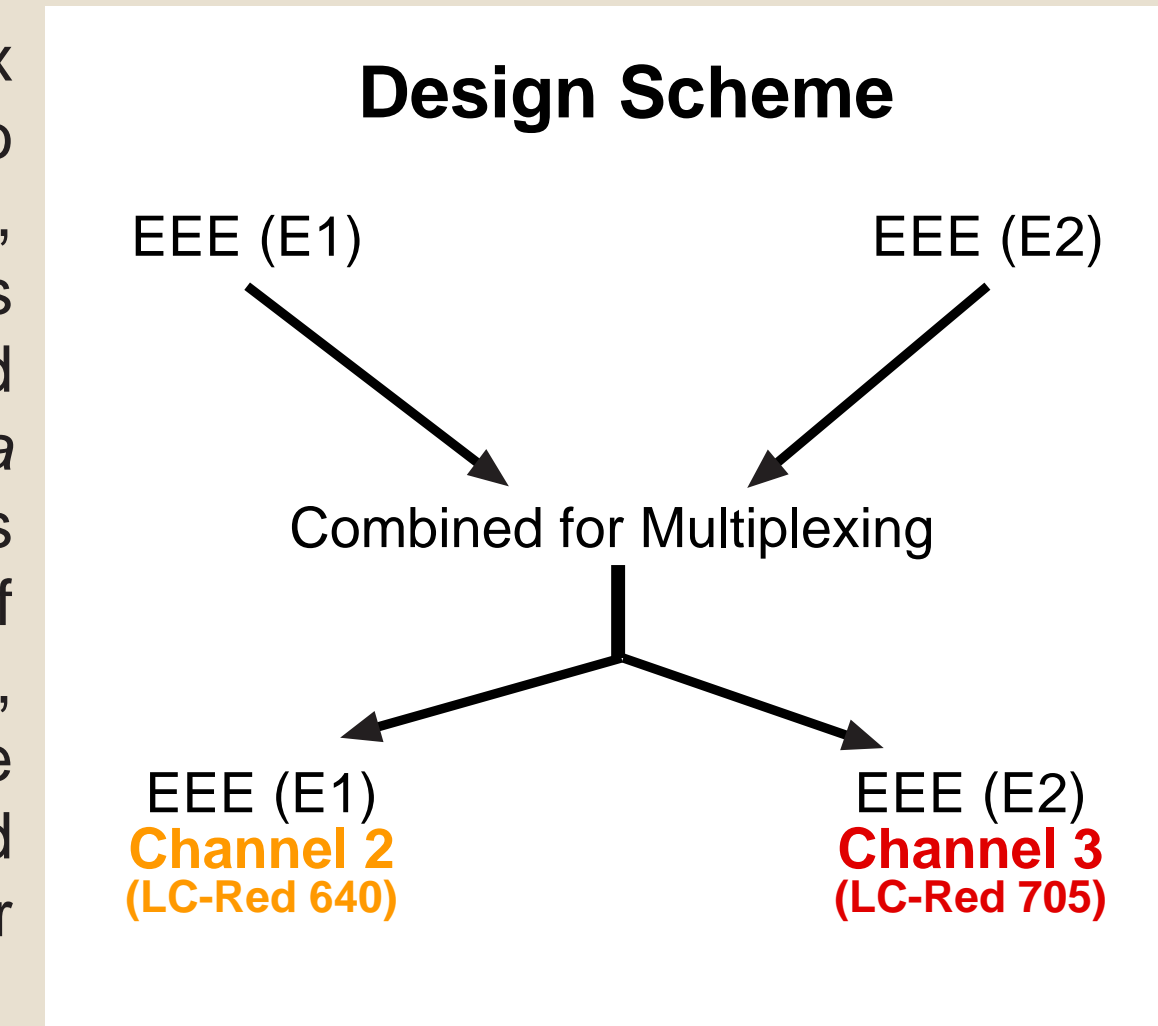
Results: The limit of detection for both gene targets was 760 fg/μl of total RNA. The experiments to determine sensitivity of this multiplex assay based upon viral PFU are ongoing.

Conclusion: The use of the R.A.P.I.D. System with this assay can decrease the time and cost of detection, allowing for increased frequency of surveillance for Eastern Equine Encephalitis.

Introduction

The development of a single-step RT-PCR multiplex assay allows for the screening and identification of two gene targets of EEE. EEE is a mosquito-borne virus, which is a member of the family *Togaviridae*, genus *Alphavirus*. The distribution of EEE is intimately associated with the distribution of the enzootic vector, *Caliseta melanura* (Blacktailed mosquito). This species occurs in the eastern United States from Canada to the Gulf of Mexico. As populations expand into endemic areas, the need for surveillance will become greater. The use of a multiplex assay reduces both the number of needed reactions and the time required to screen a large number of unknown samples.

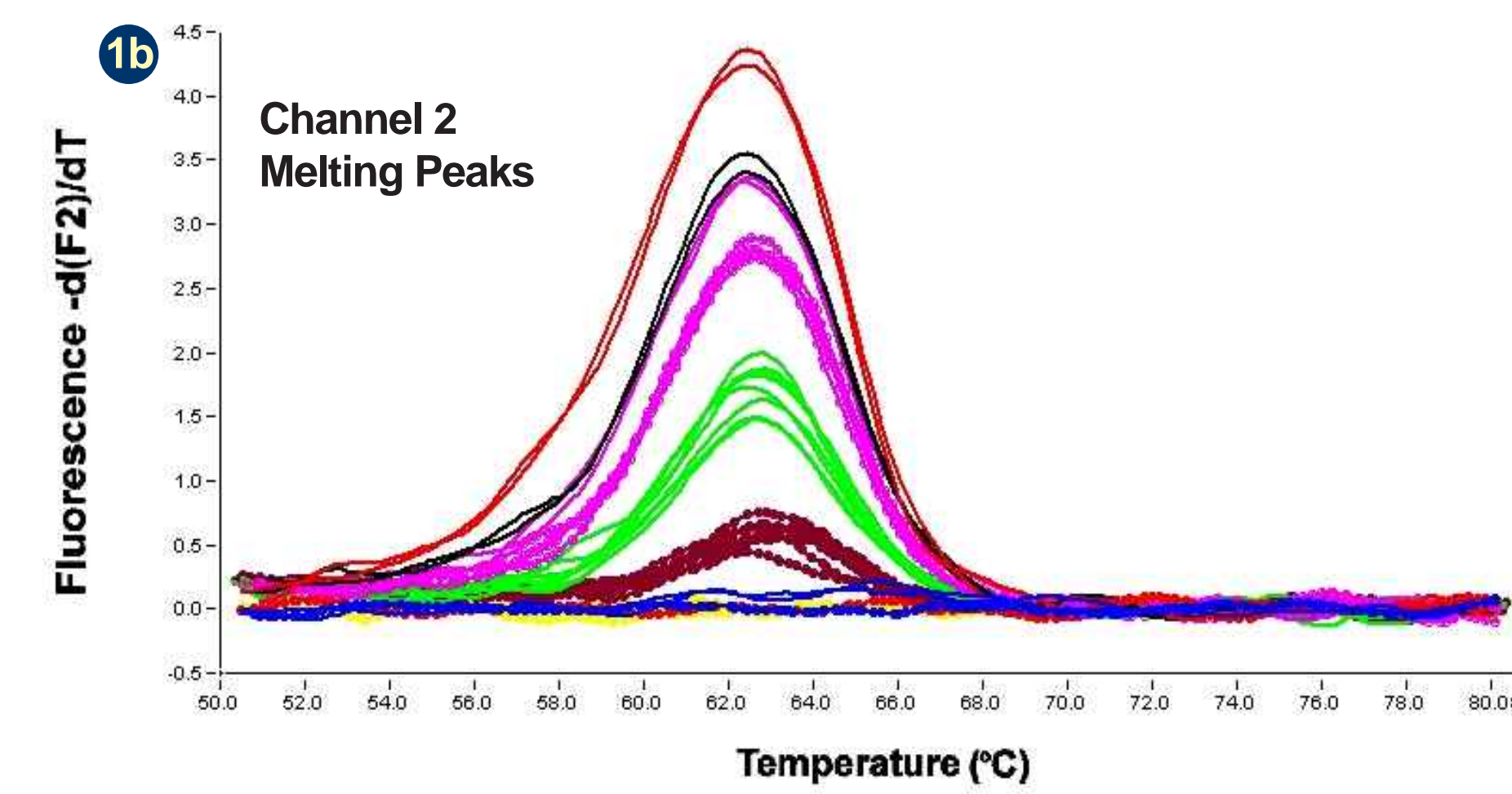
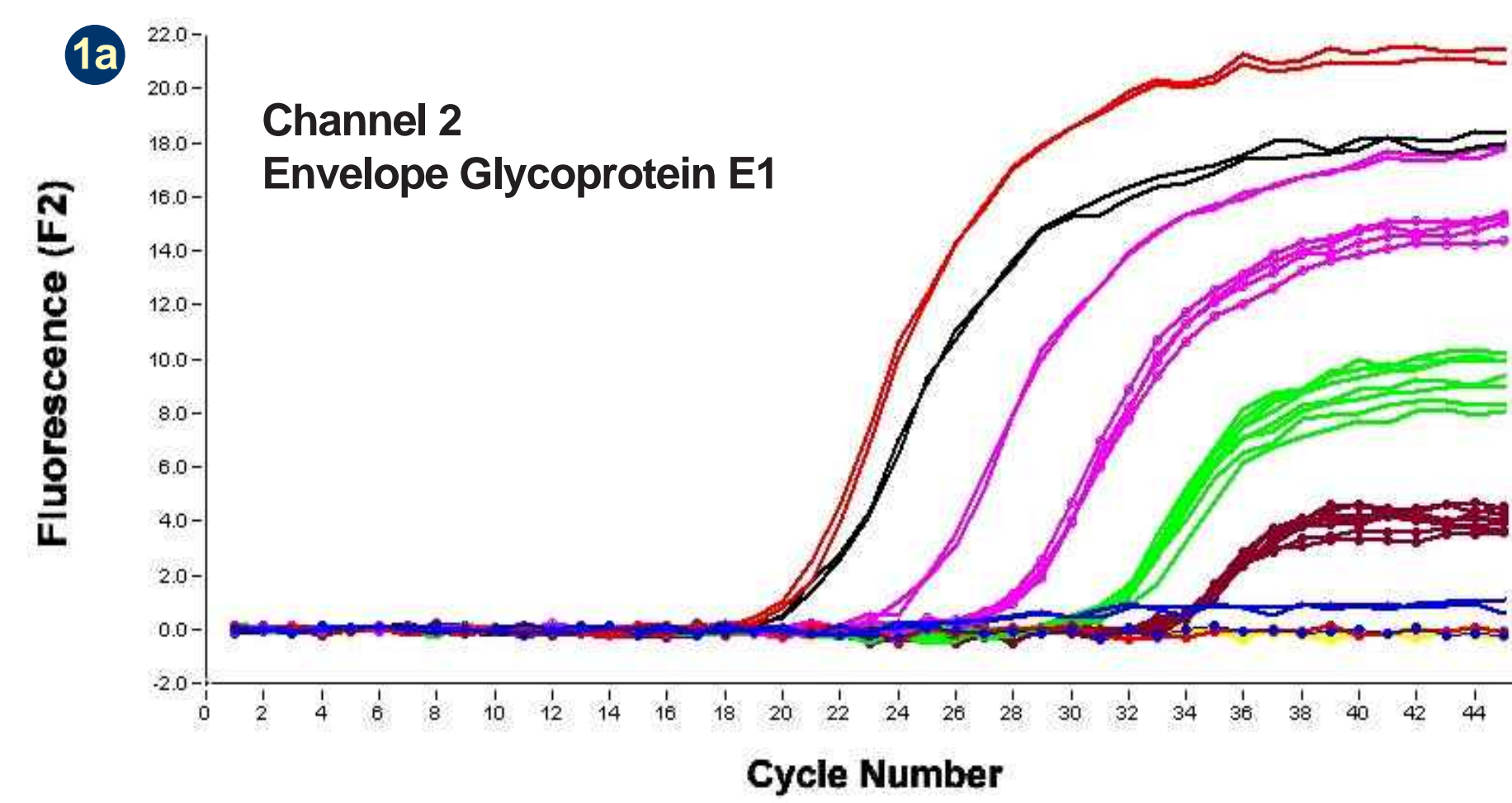
Initially two unique RT-PCR assays for EEE were designed. These single-target assays (E1, E2) were used in combination as a basis for the multiplex assay. These initial assays were designed to run under identical conditions with similar melting temperatures and were detected in channel 2 (LC-Red 640). This allowed both assays to be run simultaneously on a single R.A.P.I.D. System. In order to perform a multiplex reaction, however, some modifications were required. The detection probe for target two (E2) was changed to LC-Red 705, allowing detection to occur in channel 3. Target one, Envelope glycoprotein E1, produces an amplicon of 125 base pairs and is detected in channel 2 utilizing the dye LC-Red 640 and has a signature melting temperature (T_m) of 62.8°C. Envelope glycoprotein E2, the second target, produces an amplicon of 160 base pairs and is detected in channel 3 utilizing the dye LC-Red 705 and has a signature T_m of 64.3°C.



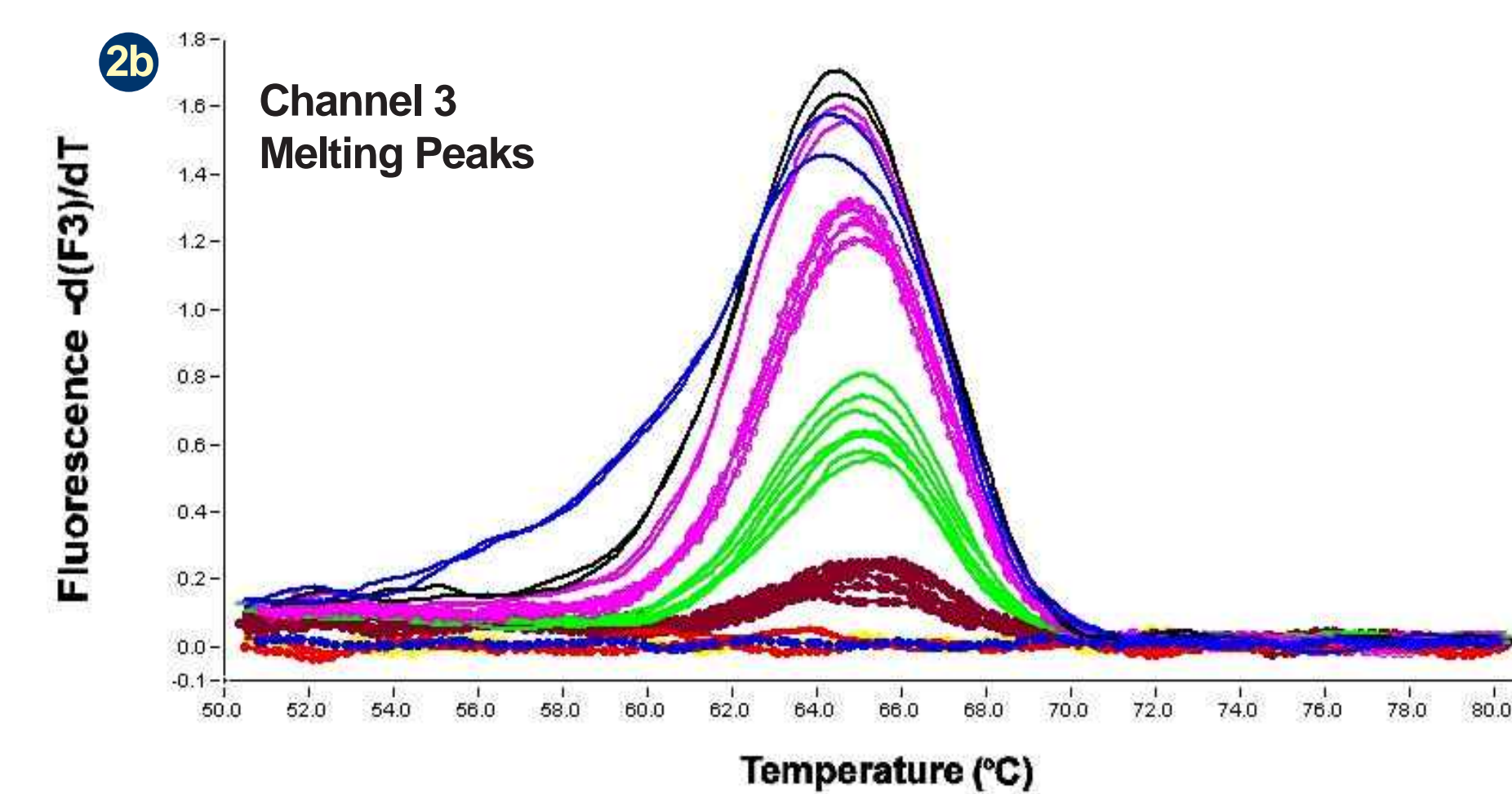
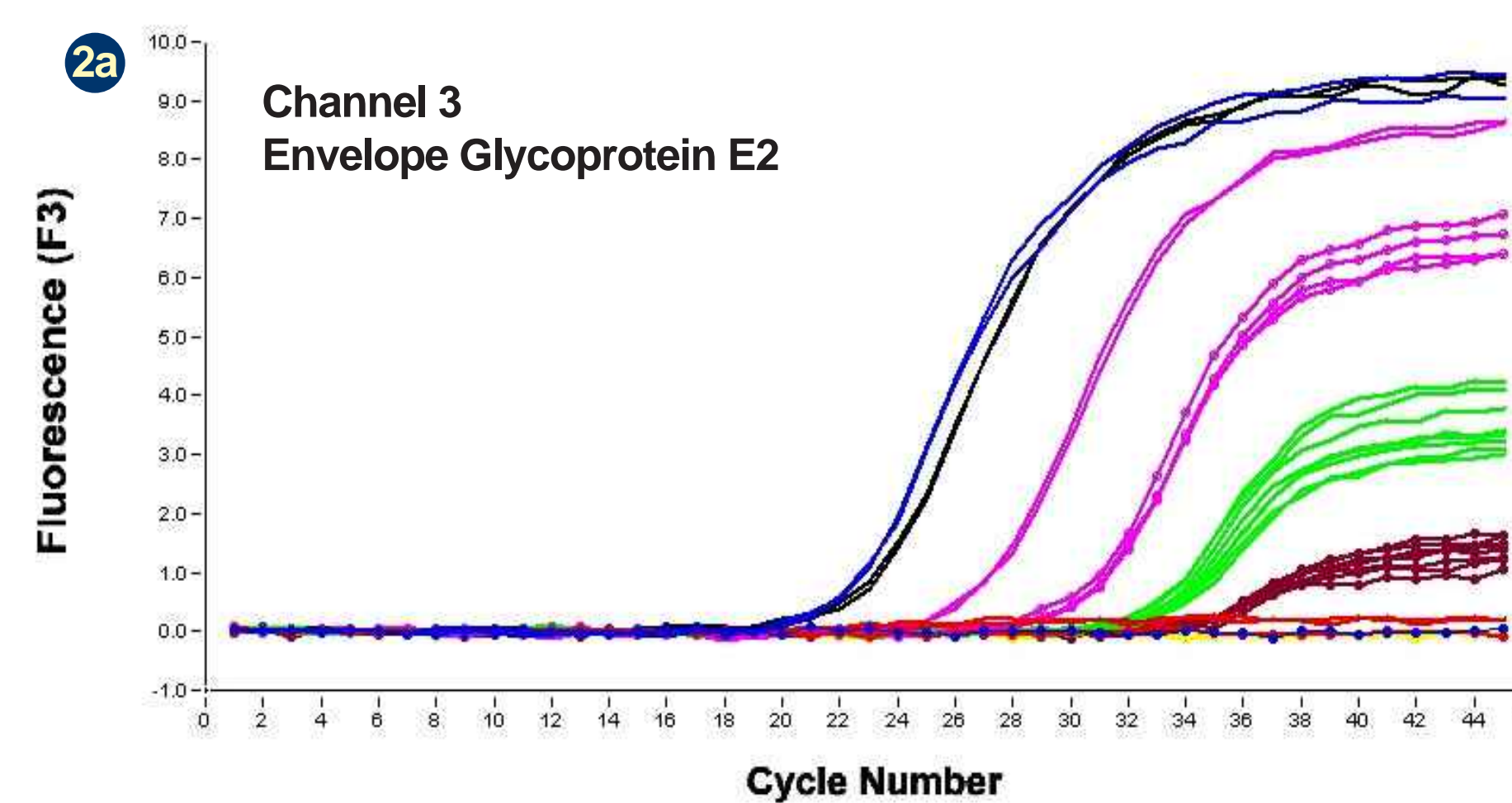
Results

Target E2 only — Target E1 only — Multiplex 7.6ng — 760pg — 76pg — 7.6pg — 760fg

Real-Time Detection of EEE Target 1 in Channel 2



Real-Time Detection of EEE Target 2 in Channel 3



Channel 2: Target E1 Sensitivity

Total RNA Concentration	Cycle Threshold (CT)	Percent Detected
7.6ng/μl total RNA	20.7	100
76pg/μl total RNA	27.4	100
7.6pg/μl total RNA	30.6	100
760fg/μl total RNA	32.5	100

Channel 3: Target E2 Sensitivity

Total RNA Concentration	Cycle Threshold (CT)	Percent Detected
7.6ng/μl total RNA	22.6	100
76pg/μl total RNA	29.7	100
7.6pg/μl total RNA	32.2	100
760fg/μl total RNA	33.5	100

Material and Methods

RNA Isolation: Samples of culture supernatant from viral infected monolayer cultures were collected and spun at 2,000 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 the EEE virus. To assure specificity of the primers, an alignment of similar genes from related organisms was performed using GenBank's Basic Local Alignment Search Tool (BLAST®). Two primer and probe sets for EEE were designed using Roche LightCycler Primer Probe™ software. The size of each gene target is 125 base pairs for E1 and 160 base pairs for E2. Detection probes were labeled with either LC-Red 640 (channel 2) or 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 contained all of the primers and probes to detect all gene targets. Buffer contained MgCl₂ buffer, dNTPs, DTT, RNaseOUT, M-MuLV (Roche), KlenTaq1 DNA polymerase, TaqStart antibody, and enzyme diluent.

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. In the case of a two-target multiplex, two different detection fluors are used. One gene target is labeled with LC-Red 640 and the second target is labeled with LC-Red 705. Color compensation is required when multiplexing on 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. In this multiplex assay we observed similar sensitivity as that observed with the single-target assays. However, the single assay and the multiplex assay were optimized on different preparations of total RNA. To date we have been unable to gain access to an *Alphavirus* panel in order to test this assay for specificity and cross-reactivity.

Conclusion

Multiplexing is an effective methodology to screen large numbers of unknown samples in a cost-effective and timely fashion. The use of this multiplex assay will allow for the simultaneous screening of two viral encephalitis targets, reducing time and cost of surveillance.

Acknowledgements

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