



Development of a Duplex Real Time RT-PCR Test for Surveillance of West Nile and Eastern Equine Encephalitis Viruses Using the Smart Cyclor

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INTRODUCTION

During the 2001 West Nile Virus season (June–October), the New Hampshire Public Health Laboratories performed over 2,200 tests, primarily on birds and mosquito pools, representing nearly 5-fold increase over the previous season. This figure is expected to increase steadily over the next few seasons. Testing for additional related encephalitis viruses is planned for the 2002 season. New probes have also become available commercially available recently with the promise of higher sensitivity and lower background.

This study was launched to simplify our testing protocols by multiplexing the high volume tests, namely, West Nile Virus (WNV) and Eastern Equine Encephalitis Virus (EEE). One EEE and 2 different WNV primer/probe combinations were tested. Probes were labeled at the 5'-end with FAM (WNV) or HEX (EEE) reporter dyes. The 3'-ends were labeled with TAMRA (WNV) or Black Hole Quenchers, BHQ (WNV and EEE). Conditions were optimized to generate comparable Ct values for both viruses even at high dilutions of the target RNA.

The developed duplex Real Time RT-PCR test will be used to test birds and mosquito pools for WNV and EEE this year.

MATERIALS & METHODS

Bird brain tissues were homogenized in 15-mL sterile Falcon conical tubes using 2 glass beads per tube and 2 mL of M4 medium (Micro Test, GA). The tubes were vortexed for 2 minutes, then centrifuged at 4 °C for 5 minutes. The supernatants were stored at -20 °C until ready for RNA extraction.

RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA). Typically, 140 µL of the supernatant were mixed with the lysis buffer (provided in the kit) and vortexed for 1 minute in the BSC.

Positive RNA controls were obtained from CDC in Fort Collins, CO.

Primers and Probes

Designation	Description	Sequence (5'-3')
WN-12	WN-1 Forward PCR Primer	cag acc acg cta cgg cg
	WN-2 Reverse PCR Primer	cta ggg ccg cgt ggg
	WN-12 Probe	6FAM-ctg cgg aga gtg cag tct cgc at-TAMRA or BHQ1
WN-34	WN-3 Forward PCR Primer	tca gcg atc tct cca cca aag
	WN-4 Reverse PCR Prime	ggg tca gca cgt ttg tca ttg
	WN-34 Probe	6FAM-tgc ccg gac cat ggg aga agc tc-TAMRA or BHQ1
EEE-12	EEE-1 Forward PCR Primer	aca ccg cac cct gat ttt aca
	EEE-2 Reverse PCR Primer	ctt cca agt gac ctg gtc gtc
	EEE-12 Probe	HEX-tgc acc cgg acc atc cga cct-BHQ2

RT-PCR Reaction Mixtures

Reactions were prepared using the TaqMan™ One Step PCR Master Mix Reagent Kit (Roche). Reactions contained the following components, unless otherwise indicated:

Reagent	Final Concentration
RNase Free Water	To 25 µL
2X PE READY MIX	1X
Forward Primer	0.1 µM
Reverse Primer	0.1 µM
Probe	0.6 µM
40X ENZYME MIX	1X
RNA	2.5–5.0 µL

Real-Time RT-PCR

Reactions were performed in the Smart Cycler (Cepheid, Sunnyvale, CA). Cycling conditions were as follows:

Program	Temperature, °C	Time, Seconds
HOLD	50	1800
HOLD	95	600
45 cycles	95	15
	60	60

RESULTS

Comparison Between TAMRA and BHQ in WNV Probes

WNV probes were labeled with FAM (5'-end) and TAMRA or BHQ (3'-end) (Bioresearch Technologies, Novato, CA). RT-PCR reaction conditions were as described in Materials & Methods. One RNA positive control was used to make 10-fold dilutions up to 10⁻⁷ in RNase-free water. Each reaction contained 5 µL of RNA. A cutoff Ct value of 39.9 was set for calling positive results. Results with Ct values equal to or above 40 were considered negative.

Our preliminary results show that both TAMRA and BHQ quenchers work well, although probes with BHQ show higher Ct values than those with TAMRA, indicating lower detection sensitivity. Both WN-12 TAMRA and BHQ probes were very sensitive, detecting RNA dilutions up to 10⁻⁵ and 10⁻⁴, respectively. WN-34 probes were one order of magnitude more sensitive than WN-12, with detection limits of 10⁻⁶ (TAMRA) and 10⁻⁵ (BHQ) dilutions.

Probe Quencher	Ct Values			
	WN-12		WN-34	
	BHQ	TAMRA	BHQ	TAMRA
RNA Dilution				
0	25.5	18.6	21.3	17.6
10 ⁻¹	28.0	22.3	24.5	20.8
10 ⁻²	31.7	26.3	28.8	24.7
10 ⁻³	35.5	31.0	32.2	28.5
10 ⁻⁴	38.4	35.6	36.4	32.9
10 ⁻⁵	40.6	37.9	39.6	36.0
10 ⁻⁶	44.9	43.0	ND	39.1
10 ⁻⁷	ND	44.3	ND	ND

Figure 1: Comparison between TAMRA and BHQ in WNV probes. ND = not detected.

Sensitivity of EEE Probe

EEE probe was labeled with HEX-BHQ, and RT-PCR reactions were carried out with 10-fold dilutions of RNA positive control as described in Materials & Methods. Each reaction contained 2.5 µL of RNA. As shown here, the EEE-12 probe is very sensitive and RNA dilutions as high as 10⁻⁸ were detected.

RNA Dilution	Ct Value
10 ⁻¹	24.2
10 ⁻²	27.0
10 ⁻³	29.2
10 ⁻⁴	31.5
10 ⁻⁵	31.8
10 ⁻⁶	32.0
10 ⁻⁷	31.6
10 ⁻⁸	28.5

Figure 2. Sensitivity of EEE probe.

WN-34 is More Suitable for Duplex Reactions with EEE-12

RNA from positive WNV and EEE birds were mixed (1:1) and 10-fold dilutions were made in RNase-free water. Five microliters of the mixture were used as template for a duplex reaction containing primers and probes for detection of both viruses. Reactions contained 0.1 µM each of WNV and EEE PCR primers. Both probes were added to reaction mixtures at a final concentration of 0.6 µM.

Results presented here show that WN-12 cannot be combined with EEE-12 in a duplex reaction. This was true regardless of the quencher used in these probes. WN-34 on the other hand was compatible with EEE-12. However, the use of BHQ quenchers caused a drop in sensitivity (high Ct values), which is in agreement with earlier observations (Figure 1). Hence, it appears that the best probe combination for this test is FAM-(WN-34)-TAMRA / HEX-(EEE-12)-BHQ. This is represented in Panel C in Figure 3.

Probe	A		B		C		D	
	WN-12	EEE-12	WN-12	EEE-12	WN-34	EEE-12	WN-34	EEE-12
Reporter	FAM	HEX	FAM	HEX	FAM	HEX	FAM	HEX
Quencher	TAMRA	BHQ2	BHQ1	BHQ2	TAMRA	BHQ2	BHQ1	BHQ2
RNA Dilution								
10 ⁻¹	41.6	21.2	ND	22.5	23.0	ND	34.8	21.4
10 ⁻²	ND	25.7	ND	27.1	29.8	25.6	36.6	26.2
10 ⁻³	ND	29.6	ND	30.7	0.5	ND	43.8	29.6
10 ⁻⁴	ND	32.8	ND	34.0	41.5	32.1	ND	33.6
10 ⁻⁵	ND	37.4	ND	39.7	ND	35.5	ND	37.6
10 ⁻⁶	ND	39.8	ND	0.0	ND	39.7	43.9	ND
10 ⁻⁷	ND	ND	ND	0.0	ND	41.7	ND	ND

Figure 3. WN-34 is more suitable for duplex reactions with EEE-12.

Optimization of Duplex RT-PCR Conditions

In Figure 3 (Panel C) WN-34 labeled with FAM/TAMRA showed much higher Ct values than EEE-12 (HEX/BHQ), although they gave similar Ct values when tested individually (data not shown). This apparent interference of EEE-12 in the WN-34 performance was overcome by titrating WNV PCR primers against the EEE primers, while keeping probe concentrations unchanged.

Reaction conditions were similar to those described in Materials & Methods except that the concentration of WNV PCR primers were varied while keeping the EEE primers at 0.1 µM. Probe concentrations were kept at 0.6 µM.

Optimum conditions were obtained in reactions 2 and 3 in Figure 4.

Reaction #	Primer Conc., µM		Ct Values	
	EEE	WNV	EEE	WNV
1	0.1	0.1	21.6	40.8
2	0.1	0.2	19.8	20.4
3	0.1	0.3	21.5	20.1
4	0.1	0.4	20.7	18.1

Figure 4. Optimization of duplex RT-PCR conditions.

Sensitivity of the Duplex Reaction

RNAs from bird tissues that have tested positive for WNV and EEE were mixed (1:1) and 10-fold dilutions were prepared in TE buffer. Five microliters of the mixture were used as template for a duplex reaction containing primers and probes for detection of both viruses. Reaction conditions were similar described in Figure 4, reaction # 3.

	A		B	
Probe	EEE-12	WN-12	EEE-12	WN-34
Reporter	HEX	FAM	HEX	FAM
Quencher	BHQ2	BHQ1	BHQ2	TAMRA
RNA Dilution				
0	22.2	24.7	20.7	22.6
10 ⁻¹	22.8	34.8	22.6	23.8
10 ⁻²	27.1	32.5	27.7	27.9
10 ⁻³	30.2	38.6	—	31.6
10 ⁻⁴	34.7	ND	35.23	38.0
10 ⁻⁵	38.0	ND	38.7	42.0
10 ⁻⁶	40.2	ND	42.3	ND
10 ⁻⁷	ND	ND	ND	ND

Figure 5. Sensitivity of the duplex reaction.

CONCLUSIONS

- ◆ TAMRA quenchers appear to offer higher detection sensitivity than BHQ.
- ◆ WN-34 and EEE-12 provided the best combination for this duplex test.
- ◆ Combining probes with different quencher dyes does not appear to create undesirable side effects, such as higher background or suppressed signal.
- ◆ The optimized reaction mixture is as shown below, with cycling conditions identical to those described in Materials and Methods:

Reagent	Final Concentration
RNase Free Water	To 25 µL
2X PE READY MIX	1X
WN-3 and WN-4	0.3 µM each
EEE-1 and EEE-2	0.1 µM each
FAM-(WN-12)-TAMRA*	0.6 µM
HEX-(EEE-12)-BHQ2*	0.6 µM
40X ENZYME MIX	1X
RNA	2.5–5.0 µL

* If error messages are seen, the probe concentration may be decreased several fold without experiencing loss of sensitivity (data not shown)

Figure 6: Optimized reaction mixture.

- ◆ A typical example of results is shown in Figure 7.

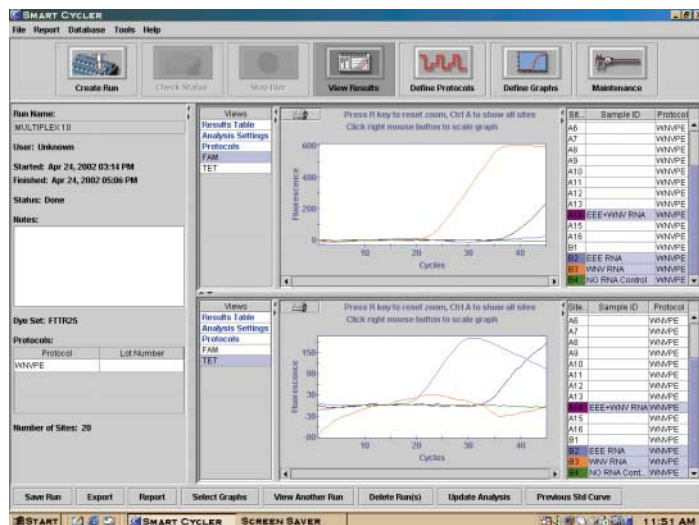


Figure 7: Duplex WNV / EEE real time RT-PCR test using the Smart Cycler. Top panel: FAM-(WN34)-TAMRA probe. Bottom panel: HEX-(EEE-12)-BHQ2 probe.

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