

Cloning of West Nile Virus Internal Control and Nucleotide Fragments Spanning the Full-Length Viral Genome for Production of Stable RNA Standards

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INTRODUCTION

- West Nile virus (WNV) is a mosquito-borne RNA virus of the family *Flaviviridae* that was first isolated in the West Nile District of Uganda in 1937 and was first detected in the northeastern US in 1999
- In June 2003, blood-collection agencies implemented investigational WNV nucleic acid-amplification tests to screen all blood donations and identify potentially infectious donations for quarantine and retrieval
- Armored RNA[®] Quant[™] is a complex of MS2 bacteriophage coat protein and *in vitro* transcribed RNA. The resulting complex is a ribonuclease resistant particle that can be used as a stable internal control in nucleic acid testing(NAT)
- The stable Armored RNA Quant particles are assembled in an *in vitro* packaging reaction
- The availability of stable WNV Armored RNA Quant fragments from known regions of the viral genome will facilitate the performance evaluation of different WNV nucleic acid tests

OBJECTIVE

Synthesize and clone an internal control (IC) and five WNV fragments spanning the full-length genome of a New York isolate for further preparation of Armored RNA[®] Quant[™], a ribonuclease resistant particle that can be used as an external or internal positive control in nucleic acid testing

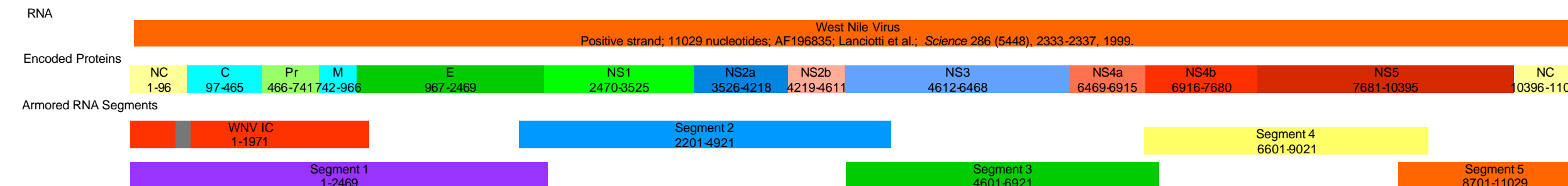
METHODS

- Five overlapping WNV fragments, roughly 1.5kb-2.5kb each, were synthesized by RT-PCR. Source of the viral nucleic acid fragments was WNV propagated in Vero cells in a BL-3 facility and subcloned into a plasmid DNA vector. The RNA fragments were within the limits of packaging for Armored RNA and spanned the entire genome of isolate NY 385-99
- A WNV Internal Control fragment containing 5' non-coding, capsid and PrM regions, with a 22-nucleotide capsid stretch substituted for the target probe binding sequence, was also synthesized and cloned
- All WNV recombinant plasmids were provided to Ambion Diagnostics, Austin, TX for custom Armored RNA[®] Quant[™] preparation
- Packaging and quality control of the transcripts was initially assayed by extraction of the Armored RNA particles and visualized on agarose gels
- For primary quantification, a colorimetric assay was performed to detect total phosphate content referenced to a NIST-traceable phosphate standard; Armored RNA Quant WNV products were subjected to heat lysis, followed by alkaline phosphatase and phosphodiesterase digestion to reduce the RNA transcripts to phosphate and nucleosides and the assay performed
- Armored RNA Quant WNV IC was also comparatively quantitated at Chiron using, as a reference, spectrophotometric readings at OD₂₆₀ nm of *in vitro* transcribed WNV internal control RNA
- Serially diluted Armored RNA Quant WNV IC in the range of 1-10,000 was added to 0.5mL plasma
- RNA from all IC dilutions was isolated and analyzed by the Chiron Target-Capture PCR assay

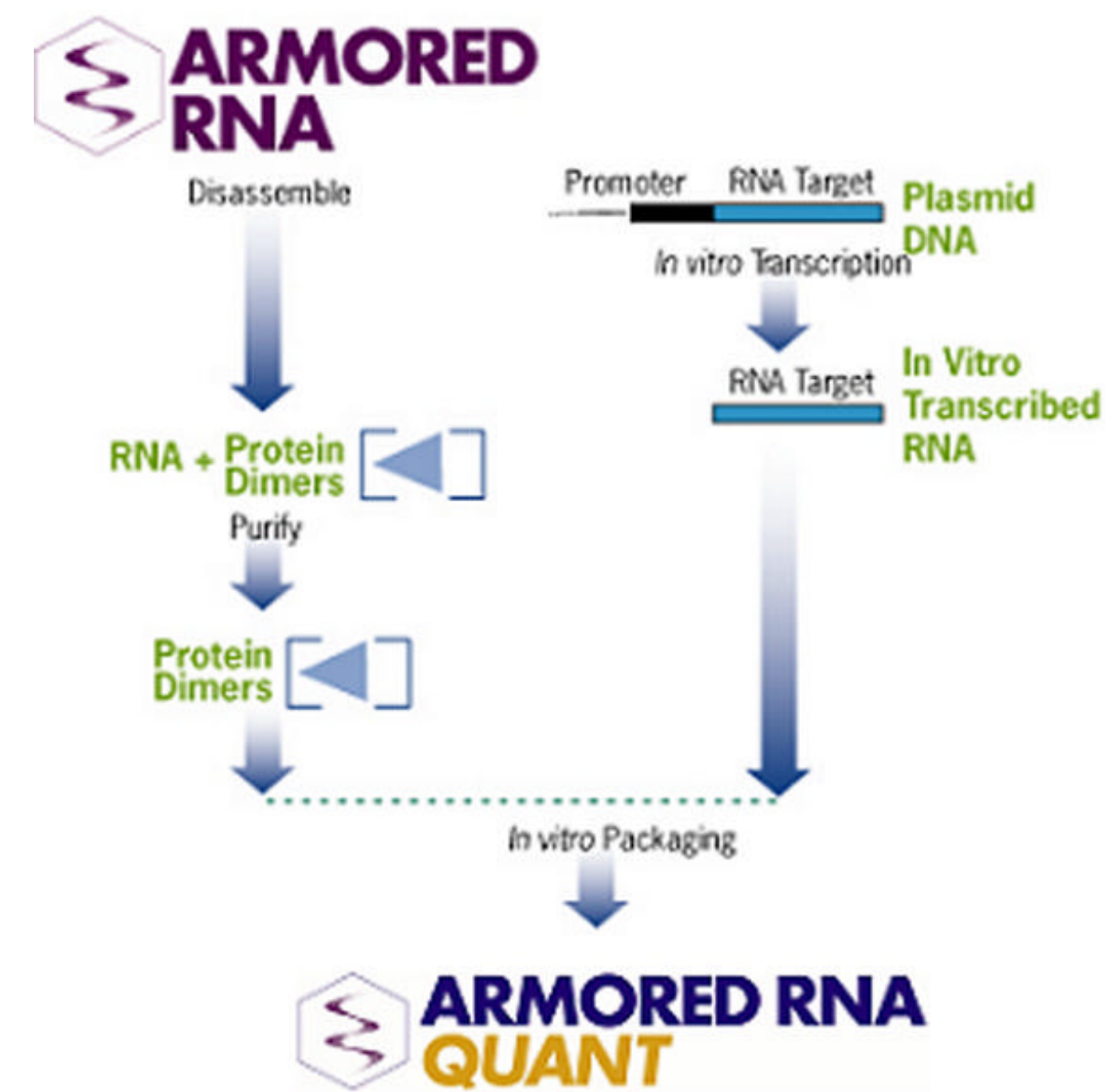
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RESULTS

WNV Genome and WNV Armored RNA Quant Segments

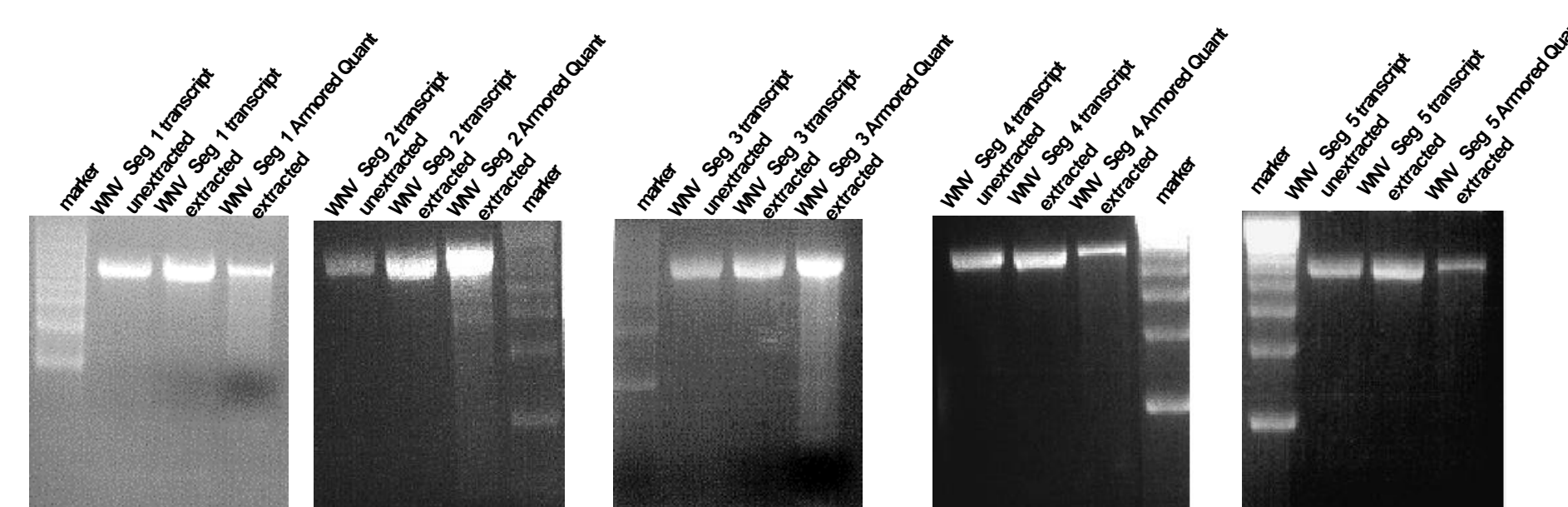


Packaging of Armored RNA[®] Quant[™]



* Armored RNA[®] technology was jointly developed by Ambion, Inc. and Genetron Diagnostics, LLC (US patents #5,677,124, #5,919,625, #5,939,262, #6,214,982, and #6,399,307). Armored RNA[®] is a registered trademark of Ambion and Genetron. For Research Use Only. Not For Use in Diagnostic Procedures.

Integrity Control of Armored RNA Quant by Gel Electrophoresis



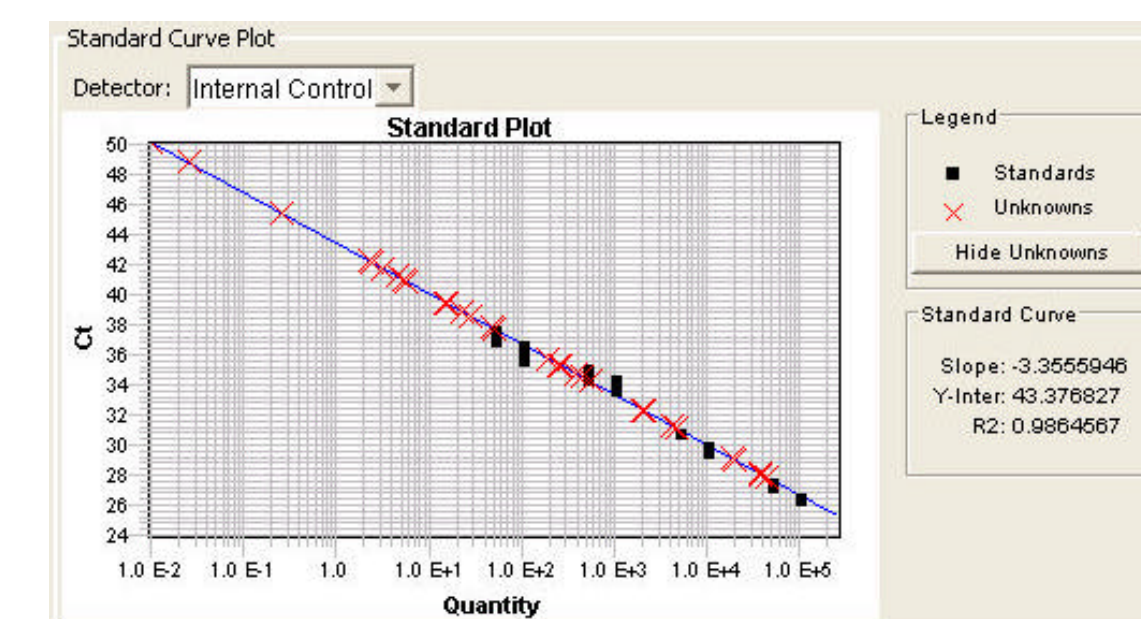
Fragment	Name	Region	Size
IC	Chiron WNV IC	nt1-966 w/ internal IC	972
S1	Chiron Segment 1	nt 1-2469	2469
S2	Chiron Segment 2	nt 2195-4915	2720
S3	Chiron Segment 3	nt 4595-6915	2320
S4	Chiron Segment 4	nt 6595-9015	2420
S5	Chiron Segment 5	nt 8695-11029	2343

Armored RNA Quant WNV Internal Control (IC)

- WNV IC is 972 nucleotides in length
- Sequence is for a 5' WNV genomic fragment that includes non-coding nucleotides, capsid and PrM regions with a target probe binding sequence substituted for the unrelated IC probe binding sequence

Comparative RNA Quantitation of WNV IC RNA and Armored RNA Quant

Standard graph WNV IC RNA



Armored RNA Copies

RNA Copies	
by phosphate assay	by spectrophotometry
100000	42173
50000	20851
10000	4614
5000	2179
1000	488
500	252
100	44
50	15
10	1
5	3
1	0
0	0

Experimental

- Standard: Internal Control WNV RNA prepared by *in vitro* transcription, quantitated spectrophotometrically and tested at 12 dilutions
- Unknowns: Test 12 dilutions of Armored RNA Quant WNV IC RNA
- Heat at 70°C for 10 min. to lyse the Armored RNA Quant particles, add RT-PCR mix, amplify and detect

Results

- Concentration dependent detection was observed with High concentration IC yielding early Cycle Thresholds (Ct)
- The phosphate assay determined concentration of Armored RNA Quant had an excellent correlation, by a factor of 2.2, with that determined by spectrophotometric readings

Uses of Armored RNA Quant WNV Internal Control (IC) in isolation and detection

- The Armored RNA Quant WNV IC was prepared for use in routine WNV Qualitative and Quantitative assays, for example, the Chiron Target-Capture PCR assay, to control for false negativity during isolation and amplification of WNV RNA samples
- In the Target-Capture PCR assay for blood screening the Armored RNA Quant WNV IC was added to the plasma sample during nucleic acid isolation
- Following isolation the IC also served as a control for amplification
- Typically the IC is used at a concentration wherein it does not compete with low copy number target.

Target-Capture PCR of Armored RNA Quant WNV (IC)

Experimental

- Serially diluted Armored RNA Quant WNV IC in the range of 1-10,000cps/rxn (by phosphate assay) was added to 0.5 mL plasma
- The IC was isolated and detected by Chiron Target-Capture PCR assay as described in "Busch et al., Screening the blood supply for West Nile Virus RNA by nucleic acid amplification testing. *New Engl. J. Med.* 2005, 353, 460-467"

Results

- *A concentration dependent Ct was observed following isolation and detection by Target-Capture PCR, indicating the suitability of IC in a routine WNV Qualitative and Quantitative assay
- The relative Ct help determine the usage of appropriate concentration per sample

Cps/rxn vs Ct*

Cps/rxn	Ct
10,000	35.33
5,000	36.61
1,000	38.49
500	41.32
100	41.67
50	41.51
10	47.59
5	50
1	50
0	50

SUMMARY AND CONCLUSIONS

- Armored RNA[®] Quant[™] technology was used to construct standards consisting of five WNV fragments spanning the entire WNV genome and an Internal Control (IC)
- Preliminary experiments to capture and quantitate the Armored RNA WNV IC:
 - Target is properly captured and detected by Target-Capture PCR assay
 - Copy numbers estimated by RT-PCR are approximately one half of those determined from a NIST-traceable standard phosphate assay by Ambion Diagnostics
- The availability of a set of WNV Armored RNA Quant synthesized from specific genomic regions will facilitate the performance evaluation of various NAT technologies