Real time RT-PCR assays for detection and genotyping of West Nile Virus lineages circulating in Africa.

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Introduction

West Nile virus (WNV) is a mosquito-borne virus belonging to the Japanese encephalitis complex of the *Flaviviridae* family including Usutu virus (USUV), Japanese encephalitis virus (JEV), Saint Louis encephalitis virus (SLEV) and Murray Valley Fever virus (MVFV) (Gubler *et al*., 2007). WNV was first isolated and identified in the West Nile region of Uganda in 1937 from a febrile female adult patient (Smithburn *et al.,* 1940). WNV is transmitted by the bite of infected mosquitoes, particularly *Culex spp* mosquitoes. Birds are considered as a main reservoir host and migratory birds and can play an important role in long distance viral spread (Hubalek and Halouzka, 1999; Hayes *et al*., 2005, Ergunay *et al*., 2015). West Nile fever outbreaks concern essentially humans, and horses as dead-end hosts (Hubalek and Halouzka, 1999; Hayes *et al*., 2005; Kulasekera *et al*., 2001; Murray *et al*., 2010). Clinical symptoms range from asymptomatic or mild influenza-like illness to severe neurological disease, which can be characterized by acute flaccid paralysis, encephalitis and meningoencephalitis (Hayes *et al*., 2005; De Filette *et al*., 2012). Since the mid-1990s, WNV outbreaks have emerged in Europe and America and WNV is now recognized as one of the most widespread flaviviruses worldwide (Vilibic-Cavlek *et al*., 2014; Hernández-Triana *et al*., 2014; DeGroote *et al*., 2014; Cox *et al*., 2015) and not restricted to tropical regions.

WNV exhibits a great genetic diversity with at least eight different lineages circulating in the world, without known specific biological properties (Mackensie & Williams, 2009; Fall *et al*., 2014). Among them, 4 (i.e. 1, 2, Koutango and a putative new lineage) are present in Africa, particularly in Senegal. Lineage 1 is the only one found worldwide, and associated with all major human outbreaks (Murray *et al*., 2010; Hernández-Triana *et al*., 2014; Anukumar *et al*., 2014).

Lineage 2 was exclusively present in Africa but since 2004, this lineage has been reported circulating in Europe, with severe cases in humans and birds (Hernández-Triana *et al*., 2014; Bakonyi *et al*., 2006). Viruses with high and low neuro-invasiveness phenotype exist in both lineages 1 and 2. Koutango and the putative new lineage are local African lineages, never isolated in humans, birds or horses (Fall *et al*., 2014; http://www.pasteur.fr/recherche/banques/CRORA/). Recently, the putative new lineage was experimentally shown to be transmitted by *Culex neavei*, which suggests a possible transmission particularly to birds, and horses (Fall *et al*., 2014). The Koutango lineage was reported more neurovirulent in mice than WNV NY99, a known virulent lineage 1 strain (Prow *et al*., 2014). These findings provided new insights into transmission and pathogenesis of these African local WNV lineages.

The report of lineage 2 in Europe, which was a local African lineage, suggests ongoing exchanges between Africa and Europe, and migratory birds that overwintered in Africa may have introduced lineage 2 into Europe (Bakonyi *et al*., 2006; Hernández-Triana *et al*., 2014). A similar migration event has been also observed for Usutu virus (Nikolay *et al*., 2013).

Interestingly, Senegal is an important stopover point for bird migration between Africa and Europe in the area of the ornithological park Djoudj on the Senegal river. All these observations suggest the potential of the African local lineages (Koutango and the putative new lineage) to spread beyond their expected geographical areas into Europe.

These different WNV lineages are not readily differentiated by serology; therefore, rapid molecular methods are required for detection and genotyping of WNV in suspect cases and potential vectors. This may have important applications in surveillance and epidemiology of African WNV lineages in Africa but also for surveillance of their emergence in Europe and other

continents. Molecular tools are available for WNV detection (Linke *et al*., 2007; Zaayman *et al*., 2009; Faggioni *et al*., 2014) but the genetic diversity of all African WNV variants has never been taken into account. Here we developed rapid molecular tools for a consensus detection of all WNV lineages (WNV consensus assay) and genotyping of the different WNV lineages reported in Africa (lineage-specific assays).

Materials and methods

Virus

Fifteen WNV and 7 other flavivirus isolates were used in this study and are described in table 1. The virus stocks were prepared by inoculating *Aedes pseudoscutellaris* (AP61) continuous cell lines for 4 days and Immuno-Fluorescence Assay (IFA) as previously described (Digoutte *et al*., 1992) was performed to assess the cell infection with WNV. Virus stocks were titrated as previously described, using PS cells (Porcine stable kidney cell line, American Type Culture Collection, Manassas, USA) (De Madrid AT and Porterfield JS, 1969). The cell culture media were used as viral stocks for RNA extraction and RT-PCR.

Primers and probes design for WNV consensus assay

In order to develop a LNA (Locked Nucleic Acid)-probe based real time RT-PCR, target regions described previously, envelope gene (E-gene) (Lanciotti *et al.,*1999) and 3'UTR (Clavero *et al*., 2006) were considered. The Envelope gene target was dismissed as it had been chosen for detection of American WNV type 1 sequences only. Using 78 sequences available from Genbank at the time of design, an amplicon was designed for the 3'UTR target region. Because of too high divergence the sequences "Rabensburg" (AY765264) and "LEIV-Krnd88-190" (AY277251) were not included in the alignments. These primers were synthesized (TIB Mol-Biol, Berlin,

Germany) and tested to identify a pair, which would not amplify any of the other Flaviviruses tested (table 2).

Primers and probes design for WNV lineage-specific assays

In order to develop lineages specific-real time RT-PCR assays, the E-gene sequences of strains belonging to each lineages (1, 2, Koutango and the putative new lineage) were considered. The different primers and probes designed (table 2) were synthesised (TIB Mol-Biol, Berlin, Germany) and tested.

RNA standard

For the consensus assay, a 1047 nucleotide fragment of the 3'UTR region was amplified using WNVUTR UP 5'-TGCTTCTGTACTTCCACAGAAGAG-3 and WNVUTR DP 5'-AGATCCTGTGTTCTCGCACC-3. For the lineage-specific assays, 727bp fragments of the E-gene were amplified with the primers WNV E-F (5′-CAACTGCCTAGGAATGAGYAACAG-3′) and WNV E-R (5′-GGCATGAGGTTCTTCAAACTC CA-3′). The obtained PCR products were ligated into pCRII (3'UTR) and pCR2.1 (E-gene) (Life Technologies, GmbH, Darmstadt) and used for *in vitro* transcription of an RNA standard with T7 RNA-polymerase (Roche, Mannheim, Germany) as described previously (Nikolay *et al*., 2014).

Determination of specificity

The specificity of the different assays was determined by testing 10 WNV strains belonging to the 4 lineages circulating in Africa (*i.e* 3 lineage-1, 4 lineage-2, 2 Koutango, and the unique strain of the putative new lineage) in comparison to other flaviviruses isolates of Dengue virus, Usutu virus, Yellow fever virus, Zika virus and Bagaza. Prior to WNV RT-PCR assays, all these isolates were tested using Pan-Flavi assay (Patel *et al*., 2013) and existing specific assays for

DENV (Wagner *et al*., 2004), YFV (Weidmann *et al*., 2010), and Zika (Faye *et al*., 2013) as described previously for amplification control.

Determination of sensitivity

Ten-fold dilutions of each *in vitro* RNA standard with known initial copy number were quantified in triplicate using the different assays. Regression curves were obtained representing the RNA copy number/reaction *vs* the threshold cycle value (Ct). The lowest RNA copy number with RT-PCR detection was considered as the analytical detection limit.

The sensitivity of viral RNA detection was also evaluated by serial 10-fold dilutions of viral stocks with known titer of each WNV type in L-15 medium (Gibco BRL, GrandIsland, NY, USA) and in human serum (Sigma Aldrich, Saint-Louis, MO, USA). To verify for the number of genome copies per plaques forming units (pfu) all supernatants were quantified using the respective WNV real time RT-PCR assays. The WNV reference strain Eg101 was used for lineage 1-specific and consensus RT-PCR assays. The WNV reference strains B956, ArD96655, and ArD94343 were used respectively for lineage 2, Koutango, and new lineage-specific assays. The assays were performed in triplicate. For the 10-fold dilutions, regression curves were obtained representing the pfu/reaction vs the threshold cycle value (Ct). The lowest titer with RT-PCR detection was considered as the analytical detection limit.

Processing of field samples and experimentally infected mosquitoes

Field mosquito pools and rodents, and experimentally infected mosquitoes (Fall *et al*., 2014) were used to test the performance of the assays. Experimentally infected mosquitoes were homogenized as previously described (Fall *et al*., 2014) and mosquito pools collected in the field were homogenized in 3 ml of L-15 supplemented with 20% of foetal bovine serum FBS. Rodent

tissues were homogenized in 3 to 4 ml of L-15 containing 5 % FBS. Viral RNA was extracted from the homogenates and analyzed by real time RT-PCR assays (see below).

RNA extraction and RT-PCR

RNA was extracted from 100 μl of each viral stock, mosquito or rodent homogenates and 10-fold dilution of WNV lineage stocks in L-15 medium or human serum using the QiaAmp Viral RNA extraction kit (Qiagen, Heiden, Germany) according to the manufacturer's instructions.

The real-time RT-PCR assay was performed using an ABI 7500 cycler (Applied Biosystems, Foster City, US) and the Quantitect Probe RT-PCR kit (Qiagen, Heiden, Germany). Reactions were performed in 25 μl reaction volume containing 1 μl diluted RNA, 0.5 μM foward and reverse primer, 0.2 μM of probe, 12.5 μl of 2× QuantiTect Probe RT-PCR Master Mix, and 0.25 μl of Quantitect RT Mix. The RT-PCR conditions were as follows: 15 min 50°C, 10 min 95°C and 40 cycles of 15 s 95°C and 1 min 60°C.

Determination of intra, inter-assay reproducibility and efficiency

Samples were extracted and amplified 10 times in the same run to evaluate intra-assay variability and in 10 different runs to evaluate inter-assay variability. The amplification efficiency of the primers was calculated from the slope of the standard regression lines ($E=10^{1/slope} -1$).

Results

Analytical specificity and analytical sensitivity

RNAs from the 10 WNV and other flaviviruses isolates were tested using Pan-Flavi, WNV consensus, and WNV lineage-specific real time RT-PCR assays developed herein. The Pan-Flavi assay detected all the isolates tested. The WNV consensus assay detected all the 10 WNV strains but initially also detected RNAs from Usutu virus strains (data not shown). Using the ARMS (amplification refractory mutation system) principle (Newton *et al.*, 1989; Weidmann *et al.*,

2003) the reverse primer was adapted to an insertion in the USUV sequences matching the 2nd

position of a newly designed WNV reverse primer (figure 1). The new WNV consensus assay

(table 2) now did not show any more cross detection of RNAs from Usutu virus strains.

To boost differentiation and therefore specificity of the individual WNV lineage-specific assays,

primers were also designed using the ARMS principle with specific matches at the 2-3 position of

the 3'-end of the primers mismatching to as many of the other strains as possible (figure 2).

These lineage-specific assays detected and correctly genotyped the respective WNV strains tested

in each lineage and did not detect other flaviviruses tested nor cross-react within other WNV

lineages tested (table 3).

The performance of the assays was tested by using 10-fold dilutions of *in vitro* RNA standards.

Five independent runs were done using the RNA standards of the consensus and each of the

lineage-specific assays (figure 3). The analytical detection limit was 10 copies/reaction for all the

RT-PCR assays (figure 3). The linear regression showed that the different curves were linear with

correlation coefficients ≥ 0.998 in all cases. Efficiencies ranged from 99% to 141% with lower

efficiencies for Koutango (103%) and putative new lineage (99%) assays, and higher efficiency

for the consensus assay (141%). The lineages 1 and 2 assays were intermediate with 129% and

121% respectively (table 4).

Viral RNA quantification

The determined titers of Eg101, B956, ArD96655 and ArD94343 (corresponding respectively to

lineages 1, 2, Koutango and new lineage viral stocks) are presented table 4. The RT-PCR

analyses on these viral stocks were done using lineage-specific assays and the copy number and

ratio copy number/pfu are presented table 4.

Ten-fold dilutions of Eg101, B956, ArD96655, and ArD94343 prepared in L-15 medium and human serum were analyzed by RT-PCR with the lineage-specific assays. The consensus assay was tested only on strain Eg101 (lineage 1) and yielded a detection limit of 0.13 pfu/reaction (Figure 4A). The lineage specific reactions yielded a detection limit of 1.3 pfu/reaction, 5 pfu/reaction, 4.49 pfu/reaction and 9.16 pfu/reaction respectively for the detection of lineage 1, lineage 2, Koutango and the putative new lineage (figures 4B-E).

The curves obtained were comparable between L-15 and human serum and linear with correlation coefficients ≥ 0.998. Efficiencies ranged from 74% to 158% for dilutions in L15, and 82% to 138% for dilutions in human serum, with higher efficiencies for the consensus assay, and lower efficiencies for lineage 2-specific assay in both L15 and human serum.

Processing of field samples and experimentally infected mosquitoes

To assess the performance of our assays, a panel of samples from experimentally infected mosquitoes, wild caught mosquito pools, and rodent tissues were used. For each experimental sample, the corresponding virus used to infect the mosquitoes was correctly detected and genotyped (table 5). The field samples naturally infected with WNV lineage 1 or Koutango, as previously assessed by classical RT-PCR and sequencing (data not shown), were correctly detected and genotyped with the lineage-specific assays in the mosquito pools or rodent tissues including brain homogenates (table 5).

Variability intra and inter-assay

The intra-assay coefficients variation (CV) ranged from 1.31 to 1.75% and the inter-assay CV ranged from 2.66 to 4.62% for all assays.

Discussion

Molecular techniques are faster, more accurate and sensitive for virus detection compared to culture methods. Here, we established molecular tools for a consensus detection of all WNV lineages and genotyping of the lineages reported in Africa. The good sensitivity of the assays allows the detection and genotyping of all WNV isolates in culture medium, human serum, as well as in field and experimental samples. After the development of the lineage specific assays new WNV lineage 2 sequences described in Europe became available (Barzon *et al*., 2013). Since they introduce a mismatch at position 3 from the 3'end of the lineage 2 specific forward primer, we suggest to use the lineage 1-specific upstream primer which is also used for Koutango for highly sensitive detection of these strains (Figure 2). However, we were not able to test these strains in the frame of this study.

Our assays allowed the quantification of WNV RNA by using quantitative RNA standards, yielding viral loads in mosquito and vertebrate samples.

Using the RNA standard, the different assays were very sensitive and able to detect 10 RNA molecules. Our RT-PCR assays exhibit therefore analytical sensitivity similar to others using WNV lineages 1 and 2 (Clavero *et al*., 2006; Barros *et al*., 2013; Del Amo *et al*., 2013).

Using viral RNA, the consensus and lineage 1- specific assay were more sensitive and were able to detect less than 2 pfu of virus. Using the same strain for the consensus and lineage 1-specific assays showed that the consensus assay is more sensitive for detection of low viral loads. This is reflected by the different ratios copy number/pfu obtained for Eg101 (56.08 with the consensus assay and 34.34 with the lineage 1-specific assay) and can be correlated with the higher efficiencies of the consensus RT-PCR assay.

Our results showed also that the different WNV lineages can be detected in human serum with slightly higher efficiencies compared to culture medium, and in vertebrate tissues, enabling the early and efficient diagnosis of these different WNV lineages from clinical samples. Finally our study revealed finally new insight on the replication of these particular WNV strains in AP61 mosquito cells. The ratios genome copy number / infectious virion (pfu) showed that new lineage strain and Koutango replication were very efficient with few genomes per pfu indicating apparently a high rate of infectious particles produced, while lineage 2 appeared to overproduce genomes indicating less efficient packaging and possibly more defective particles during its replication (Weidmann *et al*., 2011).

Conclusions

To our knowledge, the RT-PCR assays described in this paper are the first ones that allow the detection and genotyping of all reported African WNV variants.

Surveillance programs now can target all WNV variants, and our tools can be used to monitor the prevalence of WNV virus lineages in mosquito vectors and vertebrates and may be applied in diagnosis as well as in epidemiology and surveillance programs. This is particularly relevant for the surveillance of WNV lineages emerging in geographic regions where they have not previously been identified. The consensus assay is the most sensitive in all tested materials and does not cross detect any of the tested Flaviviruses and therefore is an ideal diagnostic assay covering the breadth of WNV strains worldwide.

Therefore, our tools are efficient for rapid detection of African WNV lineages, however they cannot replace conventional techniques (serology, virus isolation or molecular sequencing), which are still needed for in-depth characterization of the virus.

Acknowledgements

Author Disclosure Statement

No competing financial interests exist.

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Figure legends

Figure 1: Sequence alignment of 3'UTR sequences of WNV and USUV as indicated by accession numbers with WNV consensus assay primers and LNA-probe. Nucleotide positions refer to the sequence of reference strain Eg101 from Egypt (AF260968). AF196835 represents sequences from the USA. AY277252 represents sequences from Russia.

Figure 2: Sequence alignment of WNV E gene sequences from Africa, Senegal, Egypt, Uganda, Europe and America as indicated by strain designations with WNV lineage specific primers and probes. The forward primer WNV FP is common to lineages 1, Koutango and the European WNV2 isolates. Nucleotide positions refer to the sequence of reference strain Eg101 from Egypt (AF260968). Probe mismatches are highlighted in bold in reference to probe WNV1 P for the detection of WNV lineage 1. Mismatches introduced to the 3' ends of the primers according to the ARMS principle to enhance differentiation are highlighted in red.

Figure 3: Serial 10-fold dilutions of *in vitro* RNA standard have been tested in the corresponding real-time RT-PCR assay. Tested dilutions ranged from 1×10^8 to 0.1 copies/reaction for the RNA standard of lineages 1, 2, Koutango and new lineage-specific assays and consensus assay.

Figure 4: Serial 10-fold dilutions of virus in L-15 medium and human serum have been tested for consensus (panel A), lineage 1 (panel B), lineage 2 (panel C), Koutango (panel D) and new lineage (panel E) in the corresponding real-time RT-PCR assay.

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        10550      10560        10570       10580       10590       10600       10610       10620       10630
                             ---------+---------+---------+---------+---------+---------+---------+---------+---------+-----
AF260968 EG101       ...............................................................T................................
AF196835 US          ........................................................................................................
DQ164187 US          ..........G.............................................................................................
AY712945 US          .......................................................................G................................
AY712946 US          .......................................................................G................................
AY848697 US          .....T..................................................................................................
DQ066423 US          .....T..................................................................................................
AY660002 Mexico      ........................................................................................................
AF260969 Romania.................G......................................................................................
AF404757 Italy       ...............................................................T........................................
AF481864 Israel      ........................................................................................................
AY688948 Israel      ........................G.......................C.....T....G.........C...................................
AY532665 B956        ........................G.......................C.....T....G.........C...................................
DQ116961 Hungary...............................G................C.....T....G.........C...................................
DQ118127 Hungary     ........................................................................................................
AY603654 Ethopia     ...............................................................T........................................
DQ318019 Senegal..........................G.....................C.....T....G.........C...................................
DQ318020 CAR         ........................G.......................C.....T....G.........C...................................
AY277252 Russia      ........................................................................................................
AY490240 China       ..G.............................................................T......................................
M12294 M10103        ........................G.......................C.....T....G.........C...................................
WNVUTR FP            GTRGAYGGTGCKGCCTGC
WNVUTR P                                              CAGGAGGACTGGGTGA
WNVUTR RP                                                                                            CCAYGTAAGCCCTCAGAACCGTC
USUV KC754958        ...................................C.........T.......TT.GTTGC....GA..G.A......C...AT....
USUV KC754954        ...................................C.........T.T......T.GCCGC....GAT.G.A......C.........
USUV KC754955        ...................................C.........T........T.ACCGC....GAT.G.A......C.........
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1288      1298      1308      1318      1328      1338      1348      1358      1368      1378      1388      1398      1408      1418      1428
--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+

Lineage 1
NY 99            TTTGGCAAAGGAAGCATTGACACATGCGCCAAATTTGCCTGCTCTACCAAGGCAATAGGAAGAACCATCTTGAAAGAGAATATCAAGTACGAAGTGGCCATTTTTGTCCATGGACCAACTACTGTGGAGTCGCACGGAAA
ArD76986         TTTGGCAAAGGAAGCATTGACACATGCGCCAAATTTGCCTGCTCCACCAAGGCAACAGGAAGAACCATCTTGAAAGAGAATATCAAGTATGAAGTGGCCATCTTTGTCCATGGACCAACCACTGTGGAGTCGCATGGAAA
ArD93548         TTTGGCAAAGGAAGCATTGACACATGCGCCAAATTTGCCTGCTCCACCAAGGCAACAGGAAGAACCATCTTGAAAGAGAATATCAAGTATGAAGTGGCCATCTTTGTCCATGGACCAACCACTGTGGAGTCGCATGGAAA
ArD120040        TTTGGCAAAGGAAGTATTGATACATGCGCCAAATTTGCCTGTTCCCAAGGCAACAGGAAGAACCATCTTGAAAGAGAATATCAAGTATGAAGTGGCTATTTTTGTCCATGGACCAACCACTGTGGAGTCGCATGGAAA
AF260968 EG101   TTTGGTAAAGGAAGCATTGACACATGCGCCAAATTTGCCTGCTCTACCAAGGCAACAGGAAGAACCATTCTGAAAGAGAACATCAAGTACGAAGTGGCTATCTTTGTCCATGGACCAACCACTGTGGAGTCGCATGGAAA
WNV1 FP                         GGAAGYATTGAYACATGCCGC
WNV1 P                                                                TCCACCAAGGCAACAGGAAGAACCAT
WNV1 RP                                                                                                                                GTCCATGGACCAACCACTGTG

Koutango lineage
Koutango         TTTGGAAAGGGAAGCATTGACACATGCGCCAAGTTCACTTGCTCTCAACGCAAGCCGTGGGGAAGATCATACAGAAAGAGAACATCAAGTATGAAGTTTCAATCTTTGTGCATGGCCCAACTACTCTGGAGTCACATGGAAA
AnD95153         TTTGGAAAGGGAAGCATTGACACATGCGCCAAGTTTGCTTGCTTGCTCCAACACAAGCCGTGGGGAAGATCATCAGAAAGAGAACATCAAGTACGAAGTTTCAATCTTTGTGCATGGCCCAACTACCCTGGAGTCACATGGAAA
ArD96655         TTTGGAAAGGGAAGCATTGACACATGTGCCAAGTTTACTTGCTTGCTCAACACAAGCCGTGGGGAAGATCATCAGAAAGAGAACATCAAGTACGAAGTTTCAATCTTTGTGCATGGCCCAACTACCCTGGAGTCACATGGAAA
WNV1 FP                         GGAAGYATTGAYACATGCCGC
WNVK P                                                       TCAACACAAGCCGTGGGGAAGATCAT
WNVK RP                                                                                                                                  GTGGCATGGCCCAACTACTCTG

New Lineage
ArD94343         TTTGGAAAGGGCAGCATTGACACATGCGCGAAATTCACCTGCACAACCAAGCCATTGAAGAAGAACCATCTTCACCTGCACAACCAAGCCATTATTGAAGAAGAAAATATCAAGTATGAAGTGGCCATCTTTGTTCATGGCCCAACCAATGTGGAATCTCACGGAAA
WNVN FP                          GCAGCATTGACACATGCGCGA
WNVN P                                                       ACAACCAAAGCCATTGGAAGAACCAT
WNVN RP                                                                                                                                  GTTCATGGCCCAACCAATGT

Lineage 2
HQ537483 Greece  TTTGGAAAGGGAAGCATTGACACATGCGCCGAAGTTTGCCTGTACAACCAAAGCAACTGGATCATCCAGAAGGAGAACATCAAGTATGAGGTCGCCATATTTGTGCATGGCCCGACGACTGTTGAATCTCATGGCAA
ArD1663626       TTTGGAAAGGGGAGCATTGACACATGCGCCGAAGTTTGCCTGTACAACCAAAGCAACTGGATCATCCAGAAGGAGAACATCAAGTATGAGGTTGCCATATTTGTGCATGGCCCGACGACCGTTGAATCTCATGGCAA
AY532665 B596    TTTGGAAAGGGGAGCATTGACACATGCGCCGAAGTTTGCCTGTACAACCAAAGCAACTGGATCATCCAGAAGCAACTGGATCATCCAGAAGGAGAAACATCAAGTATGAGGTTGCCATATTTGTGCATGGCCCGACGACCGTTGAATCTCATGGCAA
ArD134613        TTTGGAAAGGGGGAGCATTGACACATGCGCCGAAGTTTGCCTGTACAACCAAAGCAACTGGATCATCCAGAACCAACTGGATGGATCATCCAGAAGGAGAAACATCAAGTATGAGGTTGCCATATTTGTGCATGGCCCGACGACCGTTGAATCTCATGGCAA
ArD177612        TTTGGAAAGGGGGAGCATTGACACATGCGCCGAAGTTTGCCTGTACAACCAAAGCAACTGGATCATCCAGAAGGAGAAACATCAAGTATGAGGTTGCCATATTTGTGCATGGCCCGACGACCGTTGAATCTCATGGCAA
WNV2 FP                       GGGAGCATTGACACATGTGC
WNV2 P                                                   CAACCAAAGCAACTGGATGGATCATCC
WNV2 RP                                                                                                                                  ACGACCGTTGAATCTCATGGC
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Figure: Standard curves for WNV Cons, WNV1, WNV2, and WNV Kout assays plotting Threshold cycle (Ct) against Copy number/reaction (log scale).

WNV Cons $y = 2.611x + 16.67$ $R^2 = 0.999$

WNV1 $y = 2.773x + 14.99$ $R^2 = 0.999$

WNV2 $y = 2.89x + 11.14$ $R^2 = 0.999$

WNV Kout $y = 3.230x + 11.04$ $R^2 = 0.999$

Threshold cycle (Ct)

Copy number/reaction (log scale)