LightMix® Kit *West Nile Virus lineages 1 and 2* EC

Cat.-No. 40-0334-32 Change: Equipped with nECT (earlier Cp values for the Control Reaction)

Real-Time-PCR Kit with reagents for the detection of *West Nile Virus* cDNA using the Roche Diagnostics LightCycler® 1.x / 2.0 / 480 II Instruments or cobas z 480 Analyzer.

Lyophilized mix of primers and probes for a total of 96 reactions with a final volume of 20 µl each. **Shipping at ambient temperature. Store protected from light at 4°C-25°C - do not freeze !**

Instructions for use with the LightCycler® 1.x / 2.0 Instruments see pages 4-5
Instructions for use with the LightCycler® 480 II Instruments and cobas z 480 Analyzer see pages 6-7

1. Introduction

West Nile Virus (*WNV*) is a coated single (+)-stranded RNA virus of the Flaviviridae family and is transmitted by mosquitoes to birds but as well as to mammals. Human infections may stay without symptoms, but the *West Nile Fever* with its influenza-like symptoms can be fatal, especially in cases with encephalitis or meningitis after the virus has passed the blood-brain barrier.

Origin of the virus is Africa while there were single reports about infections in Egypt, India and France. Since 1999 the number of infections in USA increased suddenly, accounting up to a few hundred fatal cases per year. In 2010 more than ten fatal infections occurred in South and East Europe, showing that the mosquitoes travel northwards. From the five genetic lineages in particular lineage 1 strains were associated with outbreaks and considered as emerging. Lineage 2 strains are less pathogenic and were known to confined to Africa but there were recently some severe human cases in Europe; the German authorities (PEI) excluded 2014 people who stayed in endemic areas from blood donation or request virus testing for lineages 1 and 2.

Diagnosis is based on testing of serum or cerebrospinal fluid for virus-specific antibodies (US-CDC). The viral genome can be detected by Real-Time-PCR RT-PCR, targeting the NS3 or NS5 genes, using TaqMan[1,2] or hybridization probes[3] as published already more than ten years ago.

The CCR5Δ32 deletion, associated with resistance against HIV-1, has been reported to increase the susceptibility to *WNV*, can be analyzed using LightSNiP assay rs333 or a SYBR Green based testing.[4]

[1] Detection of West Nile virus sequences in cerebrospinal fluid. Briese et al., Lancet. 2000; 355(9215):1614-5

[2] Rapid detection of West Nile Virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. Lanciotti et al., JCM 38 (2000) 4066-4071

[3] Rapid detection of the West Nile Virus. Landt et al., in Rapid Cycle Real-Time PCR. Methods and Applications. Microbiology and Food Analysis. Eds. Reischl, U., Wittwer, C. and Cockerill, F., Springer Berlin (2001)

[4] Rapid determination of the Δ32 deletion in the human CC-chemokine receptor 5 gene. Nischalke et al., 2004

2. Description

This kit provides a fast and accurate system to detect *WNV* cDNA in a nucleic acid extract; the kit includes a spiked extraction control working also as amplification Control Reaction.

A 104 bp long fragment from the NS5 gene is amplified with primers targeting virus lineages 1 and 2 and is is detected with LightCycler® Red 640 labeled hybridization probes [3].

The Control Reaction is based on a 139 bases long fragment from PhHV and is detected with LightCycler® Red 690 labeled probes. This second PCR has no visible impact on the *WNV* specific reaction and will even fail in the presence of higher amounts of *WNV* target (1,000 copies and more).

The former internal control (IC) has been changed 2014 to a spiked extraction control (sEC) to monitor successful extraction and demonstrate the ability to run an amplification reaction (no PCR inhibition). We recommend to use the 'Extraction Control' procedure; for compatibility with the former procedure the use as IC is also described. The extraction control target nECT contains Lamdba and PhHV DNA and may be used also for other LightMix kits, without adding the ECT provided with the other kit(s).
Note: EC target recovery with MagNA Pure Compact is low and target even can get lost .

Color compensation generated with the kit 40-0318 is a prerequisite to run *WNV* and control. The supplied standard row allows to determine the linear range and to estimate the quantity in unknown samples. Performance testing was made with the 'FastStart DNA Master HybProbe' using cDNA only.

3. Set Contents

3	Vials with **blue** cap containing premixed lyophilized primers and probes for 32 reactions *WNV*
1	Standard row with 6 lyophilized standards of *WNV* Lin1 from 10^1 to 10^6 target equivalents / rxn
1	Sealing foil for the standard row
1	Vial with **colorless** cap containing Positive Control DNA WNV-1 with 2.5 x 10^4 target equivalents/rxn
1	Vial with **colorless** cap containing Positive Control DNA WNV-2 with 2.5 x 10^4 target equivalents/rxn
3	Vials with **white** cap containing premixed lyophilized primers and probes for 32 reactions EC
1	Vial with **white** cap with the universal Extraction Control Target (^{n}**ECT**): 4.8 x 10^6 copies (total)
1	Vial with **black** cap containing the stabilizer for the No Template Control (NTC)
1	Certificate of Analysis (CoA) with lot-specific data

4. Additional Reagents and Items Required

	Roche Diagnostics
ColorCompensation HybProbe order n° 40-0318-00	Cat.-No. 05 997 704 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001
LightCycler® Capillaries (20 µl) (LightCycler® 1.x / 2.0 Instruments)	Cat.-No. 04 929 292 001
LightCycler® 480 Multiwell Plate 384, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 749 001
or LightCycler® 480 Multiwell Plate 96, white (LightCycler® 480 Instrument)	Cat.-No. 04 729 692 001

For use in LightCycler® 1.x Instruments with software version 3.5.3 read channel F2 instead of channel 640, channel F3 instead of channel 705 for detection. We recommend upgrading to SW version 4.1.

4.1. Optional Additional Reagents

High Pure Viral Nucleic Acid Kit	Cat.-No. 11 858 874 001
Transcriptor First Strand cDNA Synthesis Kit	Cat.-No. 04 379 012 001
LightCycler® FastStart DNA Master HybProbe	Cat.-No. 03 003 248 001

Extraction Target nECT order n° 30-0259-96

5. Product Characteristics

PCR results (activation, 50 cycles and melting curve) are obtained within 50 minutes with the capillary based LightCycler® 1.x / 2.0 Instruments and within 80 minutes with '480' plate based Instruments.

Sensitivity
These reagents detect 10 copies of *WNV c*DNA using the Roche 'LightCycler® FastStart DNA Master HybProbe' (in an exemplary system, using cloned targets as reference).

Measuring range
The linear measuring range of the assay is 10^2 to 10^6 copies of *WNV* cDNA using the Roche 'LightCycler® FastStart DNA Master HybProbe'.

Storage and Stability
- Lyophilized reagents are stable for at least 6 months after shipment when stored protected from light at 4°C-25°C. See expiry date on the product label.
- **Do not freeze** lyophilized reagents.
- Dissolved reagents are stable for at least 10 days when stored protected from light and refrigerated (4°C).

6. Experimental Protocol

Start programming before preparing the solutions. See the Instrument operator's manual for details.

6.1. Preparation of Parameter-Specific Reagents (PSR) and Control Reaction :

One reagent vial with a **blue** cap contains primers and probes to run 32 reactions *WNV*.
One reagent vial with a **white** cap contains primers and probes to run 32 reactions Control Reaction.

Check for the colored pellet, then **add 66 µl** PCR-grade water, mix (vortex) and spin down.

►**Use 2 µl** reagent for a 20 µl PCR reaction.

This solution is stable at least ten days when stored refrigerated at 4°C. Avoid prolonged exposure to light.

6.2. Preparation of Extraction Control Target (ECT):

Add **1,200 µl** PCR-grade water to the vial with **white** cap containing the Extraction Control Target

6.3. Sample Preparation:

Before extraction add **10 µl** of ECT (vial **white** cap) to the sample. Skip if IC procedure is used.
Perform nucleic acid preparation as described in the protocol of the extraction kit used (**see 4.1**).

6.4. Preparation of No Template Extraction (NTC):

Add **900 µl** PCR-grade water to the **NTC** (vial **black** cap) and
add **100 µl** of ECT (vial **white** cap).

►Use **5 µl** No Template Control DNA for a 20 µl PCR reaction.

6.5. Preparation of the Standard Row and Positive Controls

The target DNA is provided in 6 different quantities to yield from 10 to10^6 target molecules in 5 µl once resolved. Start with the lowest concentrated standard (first tube from the extended lip). Use the pipette tip to punch a hole through the sealing foil. Add **40 µl** of **NTC** (vial **black** cap) to each well. Use water if the IC procedure is selected. Mix the target DNA by pipetting the solution up and down 10 times.

Dissolve the Positive Controls (vials with **colorless** lid) in 160 µl to achieve 2.5 x 10^4 copies / 5 µl.

►Use **5 µl** standard for a 20 µl PCR reaction.

This dissolved standard row is not long-term stable and is intended for single use only. For further runs use the Positive Control. After adding the target DNA to the reaction mix, use the provided sealing foil to close the vials in order to avoid contaminations.

6.6. Preparation of the Reaction Mix

Include Positive Controls and at least one 'No Template Control' (NTC). In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions plus one reserve :

sEC Procedure	For use with the Roche FastStart Master	IC Procedure
Single reaction	**Component**	**Single reaction**
6.6 µl	Water, PCR-grade (colorless cap, provided with the Roche Master kit)	6.1 µl
2.4 µl	Mg^{2+} solution 25 mM (blue cap, provided with the Roche FastStart kit)	2.4 µl
2.0 µl	**PSR** mix (parameter specific reagents, see **6.1.**)	2.0 µl
2.0 µl	**Control Reaction** (see **6.1.**)	2.0 µl
---- µl	nECT Control Target (vials **white** cap)	0.5 µl
2.0 µl	Roche Master (red cap, for preparation see Roche manual)	2.0 µl
15.0 µl	**Volume of reaction mix**	**15.0 µl**

Table 1

To run the assay without the Control Reaction substitute ECT with 0.5 µl PCR-grade water.

Mix gently, spin down and **transfer 15 µl** of the reaction mix to a capillary or well.
Add 5 µl of sample or standard to each capillary or well for a final reaction volume of 20 µl.
Close the capillaries / attach a foil to the multiwell plate and seal, and spin down.

Start run.

7. LightCycler® 1.x / 2.0 Instruments

7.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:20	00:00:20	00:00:00	00:00:30
Ramp Rate [°C/s]	20	20	20	20	20	20	0.2	20
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0,5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Cont	None

(Melting not relevant for detection) Table 2

7.2. Data Analysis

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of the 'LightMix® Kit – ColorCompensation HybProbes'.

Perform data analysis, as described in the Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View WNV data in channel 640, Quantification mode. The negative control must show no signal.

For the Control Reaction, view data in channel 705, Quantification mode. The negative control and the low-concentrated *WNV* cDNA samples (10 to 1,000 copies) should show an amplification curve for the IC/EC with a Cp value at approximately cycle 29-33.

The provided standard row with 10^6 copies/rxn to 10 copies/rxn of *WNV* should have Cp values between cycles 17 and 35 (Cp values calculated with Second Derivative Maximum method).

For use in LightCycler® 1.x Instruments use channel F2 instead of channel 640 and channel F3 instead of channel 705 for detection.

7.3. Sample Data – Typical Results

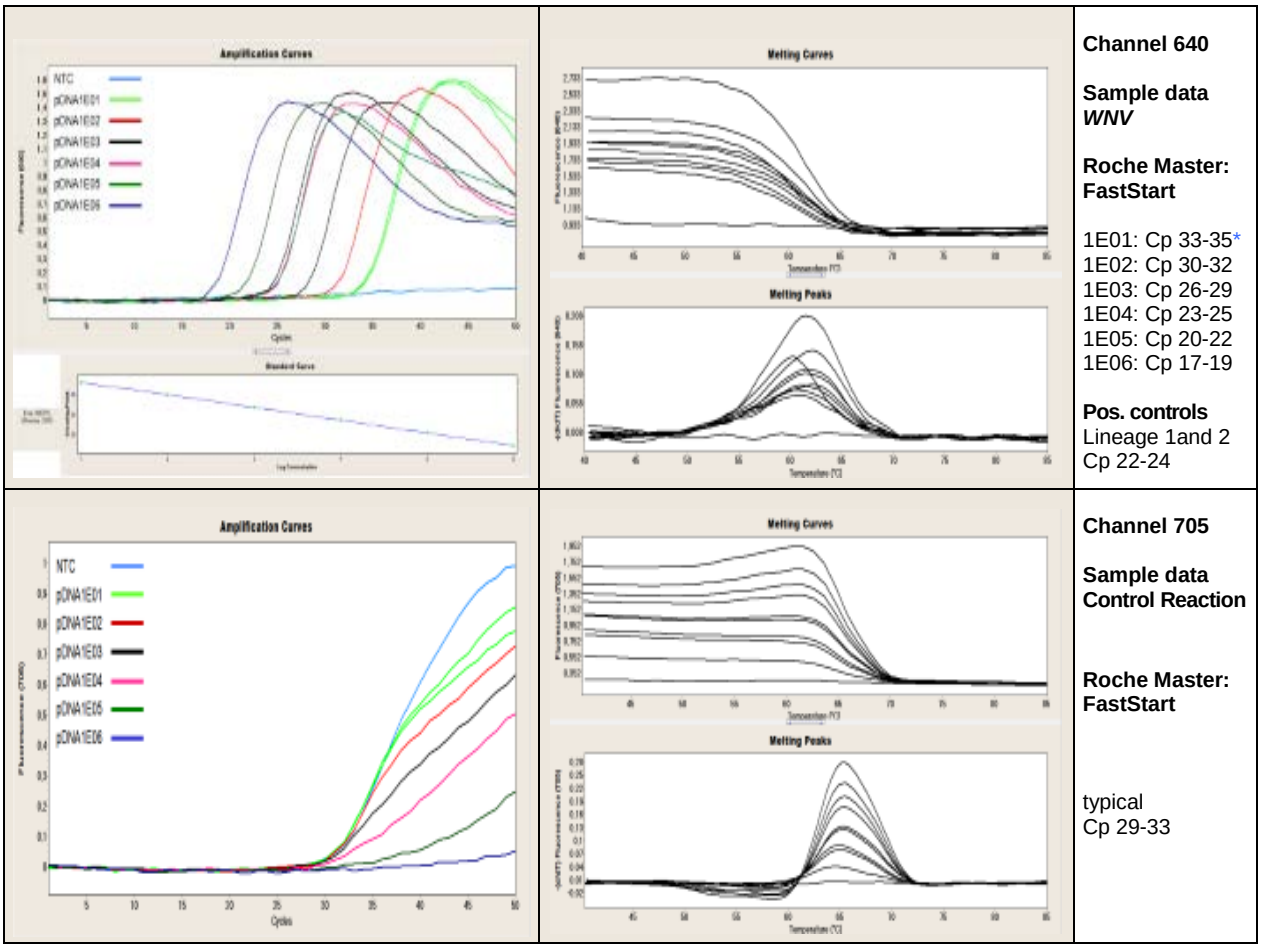

Fig.1. LightCycler® 2.0 sample data for the *West Nile Virus* detection system.

Upper panels: Left panel channel 640 quantification mode (Second Derivative Maximum) obtained with the standard row. Right panel channel 640 melting analysis obtained with the Positive Controls for lineage 1 and 2. The Tm values are not suitable for the differentiation between both lineages. Lower signals levels in the quantification screen correlating with a lower Tm in the melting analysis (> 2°C) indicate eventual variations in the probe binding region and due not interfere with the results.
Lower panels: Left panel channel 705 quantification mode (Second Derivative Maximum) for the Control Reaction. Right panel channel 705 melting analysis for the Control Reaction (not relevant for detection).

***Note**: Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

7.4. Interpretation of Data

Sample 640 *WNV*	**Sample 705** *Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	***Result*** ***(warnings)***
No amplification	Cp 29-33	amplification	negative	**Negative (not detectable)**
Cp < 37+	not relevant	amplification	negative	**Positive for *WNV***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 3 Typical analysis results with LightCycler® 2.0 Instrument, Roche FastStart DNA Master HybProbe

+ The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than for 10 copies corresponds to ~5 copies/reaction.

8. LightCycler® 480 II Instrument and cobas z 480 Analyzer

8.1. Programming

The protocol consists of four program steps

- 1: Denaturation: sample denaturation and enzyme activation
- 2: Cycling: PCR-amplification of the target DNA
- 3: Melting: melting curve analysis for identification of the PCR product derived from the target DNA
- 4: Cooling: cooling the instrument

Detection Format:
LightCycler® 480 II Instrument: 465-510, 498-640, 498-660
cobas z 480 Analyzer: 465-510, 498-645, 498-700

Program Step:	Denaturation	Cycling			Melting			Cooling
Parameter								
Analysis Mode	None	Quantification mode			Melting Curves mode			None
Cycles	1	50			1			1
Target [°C]	95	95	62	72	95	40	85	40
Hold [hh:mm:ss]	00:10:00	00:00:05	00:00:05	00:00:15	00:00:30	00:02:00	00:00:00	00:00:30
Ramp Rate [°C/s] **96**	4.4	4.4	2.2	4.4	4.4	1.5	-	1.5
Ramp Rate [°C/s] **384**	4.6	4.6	2.4	4.6	4.6	2.0	-	2.0
Sec Target [°C]	-	-	55	-	-	-	-	-
Step Size [°C]	-	-	0,5	-	-	-	-	-
Step Delay (Cycles)	-	-	1	-	-	-	-	-
Acquisition Mode	None	None	Single	None	None	None	Continuous	None
Acquisitions [per °C]	-	-	-	-	-	-	1*	-

(Melting not relevant for detection) Table 4

8.2. Data Analysis

Note: cobas z 480 Analyzer signal levels are about 50% compared to LightCycler® 480 II results.

Switch the color compensation mode on. If this mode is not enabled run the color compensation program. Follow the instructions in the manual of TIB ColorCompensation HybProbe.

Perform data analysis, as described in the LightCycler® Instrument operator's manual.

We recommend using the Second Derivative Maximum method (Automated (F" max)). The cycle number of the Crossing Point (Cp) of each sample is calculated automatically. The Fit Points method is more-error prone due to the user's influence.

View *WNV* data with Filter Combination 498-640 (498-645), Quantification mode. The negative control (NTC) must show no signal.

If the Control Reaction is used, view data in Filter Combination 498-660 (498-700), Quantification mode. The negative control and the low-concentrated *WNV* cDNA samples (10 to 1,000 copies) should show an amplification curve for the IC/EC with a Cp value at approximately cycle 28-31.

The provided standard row with 10^6 copies/rxn to 10 copies/rxn of *WNV* should have Cp values between cycles 17 and 35 (Cp values calculated with Second Derivative Maximum method).

8.3. Sample Data – Typical Results

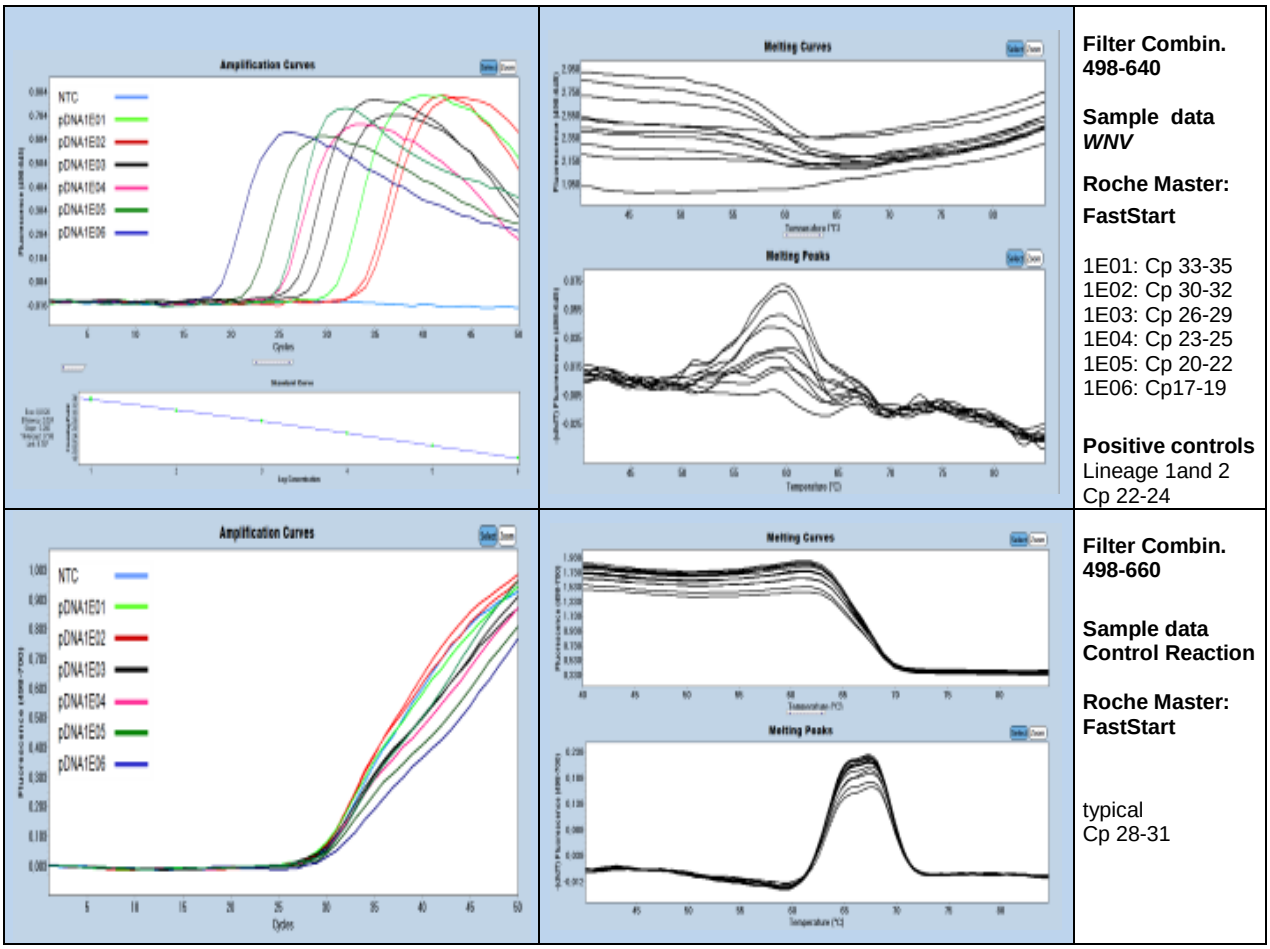

Fig.2. LightCycler® 480 II sample data for the *West Nile Virus* detection system.

Upper panels. Left filter comb. 498-640 quantification mode (Second Derivative Maximum) obtained with the standard row. Right panel filter comb. 498-640 melting analysis obtained with the Positive Controls for lineage 1 and 2. The Tm values are not suitable for a differentiation between both lineages. Lower signals levels in the quantification screen correlating with a lower Tm in the melting analysis (> 2°C) indicate eventual variations in the probe binding region and due not interfere with the results.
Lower panels: Left panel filter combination 498-660 quantification mode (Second Derivative Maximum) for the Control Reaction. Right panel channel filter combination 498-660 melting analysis for the Control Reaction (not relevant for detection).

****Note**:* Fluorescence levels depend on instrument settings and may vary. The characteristics of the curve (shape and trend of fluorescence levels) should be similar to the curve in the manual. The fluorescent curves over cycles (quantification mode) must be smooth and not zigzag shaped. The Cp values will vary from instrument to instrument by up to 2 cycles, while the distance between two dilution steps should be relative constant (delta Cp). The Cp values described in this manual (chart text) have been obtained using the supplied standard row; the entire set of curves can be horizontal shifted.

8.4. Interpretation of Data

Sample 640 *WNV*	**Sample 660** *Control*	**Channel 640** *Positive Control*	Channel 640 *Negative Control*	***Result*** ***(warnings)***
No amplification	Cp 28-31	amplification	negative	**Negative (not detectable)**
Cp < 37[+]	not relevant	amplification	negative	**Positive for *WNV***
no amplification	not detectable	amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	no amplification	not relevant	PCR failure, repeat experiment
not relevant	not relevant	not relevant	positive	Contamination, repeat experiment

Table 5 Typical analysis results with LightCycler® 480 II Instrument, Roche FastStart DNA Master HybProbe

[+] The cut off value is a recommendation only - this value has to be defined by the user. Compare with the lot specific values for 10 copies as reported in the Certificate of Analysis (CoA). 1-2 cycles later than for 10 copies corresponds to ~5 copies/reaction.

9. Conversion Factor

The amount of virus per sample (Viral Load) is usually reported in copies / ml while PCR reports copies per reaction. The conversion factor between both depends on sample and extraction volumes. Extraction starts usually from less than one milliliter and PCR does not use the total extracted volume.

The viral load (VL) can be calculated using the following general formula:

VL [copies/ml] = MV x EVF x SF

where:

VL = **Viral Load**
MV = **M**easured **V**alue [copy number per reaction]
EVF = **E**xtraction **V**olume **F**actor [Final extraction volume / PCR sample volume]
SF = **S**ample **F**actor [1,000 µl / extracted volume of clinical sample]

For example, extracting from 200µl of clinical sample results in a correction factor of 5. Using 5 µl extract from a total eluation volume of 100 µl results in a correction factor of x 20, resulting in a conversion factor of 100.

VL [copies/ml] = Measured **V**alue [copy number per reaction] **X 100**

10. Material Safety Data

According to OSHA 29CFR1910.1200, Commonwealth of Australia [NOHSC:1005, 1008 (1999)] and the European Union Directives 67/548/EC and 1999/45/EC any products which not contain more than 1% of a component classified as hazardous or classified as carcinogenic do not require a Material Safety Data Sheet (MSDS).

Product is not hazardous, not toxic, not IATA-restricted. Product is not from human, animal or plant origin. Product contains synthetic oligonucleotide primers and probes.

11. Version History

Events require changes in procedures red, mod. sequences blue

V070605	Release version (2007)
V130531	Instrument cobas z 480 included. 9. Conversion Factor included. 10. MSDS included.
V140404	Change from 16 reactions / vial to 32 reactions / vial Change Internal Control (IC) to Extraction Control (EC) 480 instruments number of acquisitions corrected to 1 Step-down cycling protocol (unified LightMix® protocol) New version detects lineage 1 and 2 virus
V150202	Amplicon size corrected (104 bp), editorial changes
V160626	Kit equipped with the universal ^{n}ECT extraction control target Cp values for the Control Reactions 1-2 cycles earlier Storage conditions 4°-25°C

Roche SAP order n° 06483445001

These reagents were developed and manufactured by TIB MOLBIOL GmbH, Berlin, Germany.