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Retro-transcription RT (two steps)

Reagents	**Producer**	**Store at**
Super Script ^{tm} II **(Invitrogen)** Riverse Transcriptase (or equivalent) - buffer First-Strand Buffer 5x: (250mM Tris-HCl pH8.3, 375mM KCl, 15mM MgCl2) - DTT 0,1 M	Invitrogen Life technologies http://www.lifetechnologies.com/order/catalog/product/18064014?ICID=search-product code : 18064-014	-24+ 6°C
Inibitor of RNase (40U/µl) [Invitrogen]	Invitrogen Life technologies AM2682 https://www.lifetechnologies.com/order/catalog/product/18064014?ICID=search-product#	-24°C ± 6°C
dNTP set - dNTP mix: dATP, dCTP, dGTP, dTTP (10 mM (2,5 mM each dNTP) [Roche]	Roche Life Science code: 11969064001 http://lifescience.roche.com/shop/en/global/products/deoxynucleoside-triphosphate-set-3651172-1	-24°C ± 6°C

Composition	pd(N)$_6$ random primer [Roche : http://lifescience.roche.com/shop/products/primer-random] code: 11034731001
Preparation	Suspend random primer at final concentration of 2500 ng/µl;
Store at	-24+ 6°C

Denaturation of secondary structure of extracted RNA

Add to 5µl of RNA extracted, 2 µl of Random Primer [2500 ng / µl] and 5,5µl di water RNase free

1. Put tubes at 70°C for 10' and at the end of this period quickly put tubes in ice.

To each tubes (with RNA denaturated) add 7,5 µl of retro-transcription MIX:

REAGENT	**FINAL concentration**	**Volume**
buffer 5X First Strand Buffer (*Super Script II™ Invitrogen)*	1x	4 µl
DTT 0,1 M (*Super Script II™ Invitrogen)*	0,01 M	2 µl
dNTPs (2,5 mM)	0,5 mM	1 µl
Rnase inibitor (40U/µl)	0,5U/ µl	0,25 µl
Super Script™ II	2,5 U/ µl	0,25 µl
Total volume Mix RT		7,5 µl

ANNEX 5: Diagnostic scheme West Nile and Usutu virus

The cDNA synthesis protocol:

STEP	**Temperatura**	**Tempo**	**step**
1st step	42 ° C	90 minutes	1
Denaturation	94 ° C	5 minutes	1

After retro-transcription the cDNA could be conserved at 5 ± 3°C until Real Time Reaction or in -24± 6°C for longer time (up to 2 years).

Real Time PCR

REAGENTs	**FINAL Concentration**	**VOLUME** **x samples**
Master MIC 2X JumpStart™ *(SIGMA D7440)* *http://www.sigmaaldrich.com/catalog/product/sigma/d7440?lang=it&region=IT*	1x	10 µl
$MgCl_2$ 25mM	2.5mM	2 µl
Mix Primer and probe*	10pmol (primers) 7pmol (probe)	2.2 µl
water RNasi free		2.8 µl
Total MIX volume		17 µl
cDNA		3 µl
Total reaction volume		20µl

*

Normally we prepare a primer and probe mix in this way:

Re-suspend primer and probe at 100pmol/µl (µM).

70 µl of probe
100 µl of each primer (200 µl on total)
630 µl of water (PRC grade).

Doping this you have 1000ml of primer and probe mix that have to be divided is different tubes (aliquots) and stored ad -20°C.

We buy Primer from :

https://ecom.mwgdna.com/register/index.tcl

we ask for unmodified oligos

https://ecom.mwgdna.com/services/oligo/oligonucleotides.tcl?ot=OLIGO_ALC_UNMOD

HPSF purification and concentration of 0.2 µmol

ANNEX 5: Diagnostic scheme West Nile and Usutu virus

Probe

Sigma \ oligos and peptides \ dual label probes

https://www.sigmaaldrich.com/configurator/servlet/DesignCenter?btnOpen_3.x=1

normally I order 3 OD per probe.

ANNEX 5: Diagnostic scheme West Nile and Usutu virus

Real Time PCR profile:

STEP	**Temperature**	**Time**	**cycles**
Denaturation and enzime activation	95 ° C	3 minutes	1
Denaturation	94 ° C	10 seconds	50
Annealing	58 ° C	20 seconds	
Elongation	72 ° C	20 seconds	

Diagnostic scheme WN- USU

1) Screening Real Time PCR (Tang et al. 2010)

WN real time

WN10533 10552 - AAG AGT AGA TTG CGG TGC TG

WN10625 - 10606 CGG AGA TTC TGA GGG CTT AC

Probe WN10560 - 10579 CTC AAC CCC AGG AGG ACT GG- BHQ1

On the positive (CT less or equal to 45) do the following 2 Real Time PCR to confirm the positivity:

WN 1 and 2 Eiden et al.2010 (method OIE)

FLI - WNF5 -F GGGCCTTCTGGTCGTGTTC

FLI - WNF6 -R GATCTTGGCYGTCCACCTC

FLI - WNF -Probe FAM- CCACCCAGGAGGTCCTTCGCAA- TAMRA

WN 1 and 2 INMI Spallanzani (method used by medical doctors has a significantly lower sensitivity than Tang or Eiden and it may be negative on vet-samples)

Primers : WN12ProC FW1 ATT GCC CGT TTT AGC TTG ATA A

WN12ProC FW2 GCC CGT TTT ATA ATA AGC TTG AC

WN12ProC RW ACA TTA TGC CTG GAG CTG TTT C

WN12ProC RW2 TTA ACA TGC CAG GAG CTG TTT C

WN12ProC PROBE Probe FAM- TAGACATCGAGATCTTCGT - BHQ1

To exclude that the positivity is not due to USU , especially if the Ct is between 35 and 40 proceed with the Real Time PCR Cavrini et al. and traditional PCR Manarolla et al., that is also useful for sequencing the USU , if positive.

USU Cavrini et . 2010

USU -F - 5 -3 AAAAATGTACGCGGATGACACA ,

USU - 5 - R -3 TTTGGCCTCGTTGTCAAGATC

USU -P - 5_ - 6famCGGCTGGGACACCCGGATAACC tamra_3

USUTU G. Manarolla et al. (2010)

Usu1155f CTAGCCACTGTCTCATATGT

Usu1600r ATGTAGTATGCCTCGGTGTT

2) To all positive in real time PCR (WN , USU or both) proceed with traditional PAN-flavivirus PCR of Scaramozzino for sequencing.

cFD2 - GTGTCCCAGCCGGCGGTGTCATCAGC

MAMD - AACATGATGGGRAARAGRGARAA

On WN confirmed positive samples try to obtain Lanciotti et al. 2000 for sequencing.

WN traditional

WN233 TTGTGTTGGCTCTCTTGGCGTTCTT

WN600c CAGCCGACAGCACTGGACATTCATA

Circuits lab in which you participated with the previous diagnostic scheme between 2009 and 2013

From 2009 - 2013 : circuits organized by the Center of Disease Control and Prevention , National Center for Infectious Disease , Division of Vector- Borne Infectious Disease , Fort Collins, Colorado , USA. A circuit for a year, with everyone always satisfactory results .

2010: Circuit interlaboratory ORGANISATION OF SAFETY + within the European project EPIZONE by the reference laboratory for the eutopero WN , EURL for Equine Diseases, ANSES Maisons- Alfort, France , with fully satisfactory results .

2013 : Circuit interlaboratory ORGANISATION OF SAFETY + eutopero by the reference laboratory for the WN (EURL for Equine Diseases, ANSES Maisons- Alfort, France) circuit organized by the National Reference Laboratories on equine diseases to which we were invited to participate despite we were not the center of reference in matter, also in this case with fully satisfactory results

2013 : Circuit organized by the National Reference Centre IZSAM with fully satisfactory results , reported as informal anticipation waiting for the final report .

DECISIONS TREE

If Tang negative -> negative sample.
If Tang and Eiden and / or Spallanzani POSITIVE -> WN positive sample, and proceed with flavivirus sequences
If Tang and Cavrini POSITIVE (Eiden and Spallanzani negative) ->USU positive sample, do flavivirus sequences

It can happen that the samples are positive for both viruses, in this case will be positive all the Real Time PCR.

If you want to try to typing lineage 1 and lineage 2 Del Amo 2013 (Journal of Virological Methods 189 (2013) 321– 327) could work.

References

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