



ADIAVET™ A/H1N1 (2009) REAL TIME

TEST FOR THE DETECTION OF THE PANDEMIC INFLUENZA VIRUS
A/H1N1/04/2009 ON PIGS BY REAL-TIME ENZYMATIC AMPLIFICATION (RT-
PCR TEST)

Reference:

ADI441-50 (50 reactions)
ADI441-100 (100 reactions)



NOTE

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English version

NE441- 09

2020/05

ADIAVET™ A/H1N1 (2009) REAL TIME

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I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2013/11	NE441-05	Correction	The supplier changed NucleoSpin® RNA II designation for NucleoSpin® RNA, in page 6, § III-4.
2013/11	NE441-05	Technical change	Addition of optional 10 µl/ml β-mercapethanol to liquid biological samples, in page 9, § V-1.
2013/11	NE441-05	Correction	The supplier changed RA2 designation for RAW2, in page 12, § IV-3.
2013/11	NE441-05	Technical change	Addition of optional 10µl/ml β-mercapethanol f to liquid biological samples, in page 11, § V-3.
2013/11	NE441-05	Technical change	Addition of definitions, in page 16, § VII-1.
2015/12	NE441-06	Technical change	Removal of reference ADI441-100 (100 reactions)
2015/12	NE441-06	Technical change	Addition of « Index of Symbols » section, in page 18, § VIII.
2016/07	NE441-07	Administrative	Changing logos
2016/07	NE441-07	Administrative	Biosearch legal mention
2016/07	NE441-07	Administrative	Addition of table "Analysis options according to the specimen" §I.3.
2016/07	NE441-07	Technical change	Addition of the optional nature of the β-mercapethanol (10µl/ml) to liquid biological samples, in page 13, § V-5.
2020/01	NE441-08	Technical change	Addition of a NF-Water tube in the kit. Addition of ADIAMAG kit using magnetic beads
2020/05	NE441-09	Technical change	Addition of reference ADI441-100 (100 reactions)

II. General information

1. Purpose of the test

ADIAVET™ A/H1N1 (2009) REAL TIME kit is intended to detect the A/H1N1/2009 Pandemic Influenza Virus (A/H1N1 Virus) using real-time Polymerase Chain Reaction (PCR) technology from nasal swab, from allantoic or bronchial fluid and tissue specimens of pig, as well as from viral culture.

2. Pathogen

The swine flu is an important pig farm disease because it could lead to severe respiratory symptoms. It is generally characterized by a high number of infected animals but a low mortality level. The disease is due to a virus of the Influenza type A genus. The first isolation of an influenza virus on pig occurs in 1930 (Influenza type A, H1N1). In opposition to the human flu, Swine flu could spread in pig farms all over the year. Three sub-types of Influenza type A virus can usually infect pigs: H1N1, H3N2 and H1N2. H1N1 and H1N2 are enzootic in Europe.

Since the April 2009, the pandemic flue due to the new variant of the influenza virus A(H1N1) rapidly spread in the worldwide human population. It is then important, for animal and human health, to identify among influenzal infections that periodically affect pig farms, those related to the pandemic influenza A/H1N1 in order to limit the disease spreading.

The detection of the virus is usually performed by viral culture on nasal swabs. Viral culture as well as diagnostic tests based on the detection of nucleoprotein antigen (such as immunofluorescence staining assay) can be carried out on lung sample. Viral culture has to be performed at an early stage of the disease because the virus persists only few days at the lesions level.

Real time PCR can be an interesting, specific and sensitive alternative method to obtain a result in one day if nasal swabs are taken on the early first clinical signs (exhaustion, fever, loss of appetite, respiratory difficulties).

3. Description and purpose of the test

This test is based first on the reverse transcription (RT) of RNA into complementary DNA. Then, cDNA is amplified (PCR) by a DNA polymerase using specific primers. Both enzymatic reactions occur in the same tube (One-step RT-PCR).

Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonuclease technology).

The ADIAVET™ A/H1N1 (2009) REAL TIME kit enables the simultaneous detection of:

- pandemic influenza virus A/H1N1/04/2009 (probe labelled in FAM),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous RNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE validated the test using RNA purification kits (Bio-X Diagnostics, Qiagen, Macherey-Nagel). Other purification kits can be used if they have been validated by the user.

Analysis options according to the specimen:

Specimen	Individual analysis	Pool of sample is possible*, up to
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Nasal swab	☒	3
Allantoic or bronchial fluid	☒	☒
Tissue (lung)	☒	☒
Viral culture	☒	☒

* It depends on the epidemiological case and on the quality of the specimen. Not recommended for the diagnostic in case of clinical signs.

This test is recommended in France by the ANSES (French agency for sanitary security of foods) to detect the A/H1N1 (2009) Influenza pandemic virus in pig farms.

III. Material and reagents

1. Reagents provided with the kit

REF ADI441-50

A5	amplification solution	1 x 1000 µl tube with green cap (a ready-to-use reagent)
A/H1N1 CTL+ ...	positive control <i>Pandemic Influenza Virus A/H1N1/04/2009</i>	1 tube with purple cap (to reconstitute)
SIV CTL-	Negative control Swine Influenza Virus	1 tube with purple cap (to reconstitute)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

REF ADI441-100

A5	amplification solution	2 x 1000 µl tubes with green cap (a ready-to-use reagent)
A/H1N1 CTL+ ...	positive control <i>Pandemic Influenza Virus A/H1N1/04/2009</i>	1 tube with purple cap (to reconstitute)
SIV CTL-	Negative control Swine Influenza Virus	1 tube with purple cap (to reconstitute)
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

Package insert downloadable from www.biox.com

2. Validity and storage

On receipt, the kit should be stored at <-15°C.

It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.

Do not defrost reagents more than 3 times.

Realtime reagents are susceptible to light: store them in the darkness.

The A5 reagent is ready to use for PCR reaction.

Do not mix reagents of two different batches.

3. Use of controls

A. A/H1N1 CTL+

Add **200 µl** of **NF-Water** to the **A/H1N1 CTL+** tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Use this solution like a sample RNA extract. Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, we recommend to use **5 µl** of **A/H1N1 CTL+** in one of the wells. A standard range could be obtained from tenfold dilution (10⁻¹ to 10⁻⁵) of the positive control solution.

B. SIV CTL-

Add **200 µl** of **NF-Water** to the **SIV CTL-** tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Use this solution like a sample RNA extract. Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, we recommend to use **5 µl** of **SIV CTL-** in one of the wells.

4. Equipment required but not supplied in the kit

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality.
- Class II Microbiological Safety Cabinet
- Centrifuge for microtubes, 96-wells plate
- Universal laboratory grinder Mixer Mill or Fast Prep
- Etuve, heating baths or block heaters
- Instrument for homogenous mixing of tubes
- 96 wells plates' agitator
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Multichannels pipette 1000 µl
- Nuclease-free filter tips

- Nuclease-free microtubes: 1.5 ml and 2 ml
- 96-well plates (ELISA-like)
- Powder-free Latex gloves
- Metal beads 3 mm
- 96-100% ethanol solution
- Nuclease-free water
- Sterile saline water (NaCl 8.5 g/l)
- β -mercaptoethanol 14.5 M
- MEM medium + antibiotic (penicillin 100 IU/ml and streptomycin 100 μ g/ml)

- **RNA extraction kit (individual columns)**
 - RNeasy® Mini Kit (Qiagen, 50 extractions: ref. 74104 or 250 extractions: ref. 74106)
 - or
 - QIAamp® Viral RNA (Qiagen, 50 extractions: ref. 52904 or 250 extractions: ref. 52906)
 - or
 - NucleoSpin® RNA (Macherey-Nagel, 50 extractions: ref. 740955.50 or 250 extractions: ref. 740955.250)
 - or
 - NucleoSpin® RNA Virus (Macherey-Nagel, 50 extractions: ref. 740956.50 or 250 extractions: ref. 740956.250)

- or

- **RNA extraction kit (plate columns)**
 - Nucleospin® 96 Virus (Macherey-Nagel, 2x96 extractions: ref. 740691.2 or 4x96 extractions: ref. 740691.4)
 - MN Square-well Block (Macherey-Nagel, 4 plates: ref. 740476), optional

- or

- **RNA extraction kit (8-wells extraction strips)**
 - Nucleospin® 8 RNA (Macherey-Nagel, 12x8 extractions: ref. 740698 or 60x8 extractions: ref. 740698.5)

- or

- **Automated DNA/RNA extraction kit using magnetic beads**
 - ADIAMAG (Bio-X Diagnostics, 200 tests: ref. NADI003)

IV. Recommendation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiogene has elaborated this PCR test with the use of extraction kits from Bio-X Diagnostics, Qiagen, Macherey-Nagel. Other extraction kits can be used with a previous validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

Some kits include and/or need the use of toxic reagents. These reagents should be use with gloves and into chemical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. Ensure the accuracy and precision of the micropipettes used. The quality of the obtained results depends upon rigorous respect of good laboratory practices.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification.

Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using fractions of demineralised and saline water and to autoclave them twice 25 minutes at +120°C or once 60 minutes at +121°C. Take a new fraction for each new manipulation to avoid contamination.

2. Storage of samples and DNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at <-15°C.

Extracted RNAs are quite sensitive molecules. Extraction is made at room temperature and should be performed as fast as possible to avoid degradations. We then recommend to read the entire protocol before starting the test and to rigorously respect it. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for few hours, then at <-15°C.

3. Samples preparation

A. Nasal swab

Add **2 ml** of MEM medium + antibiotic (in order to allow an ulterior viral culture) or of sterile saline water in the tube of the swab.

Homogenize the swab.

Transfer the liquid in a 2 ml-microtube.

Press the swab to collect as much liquid as possible.

Take **200 µl of sample**.

NB1: Store the rest of the liquid at -70°C +/- 10°C for a new analysis or for a viral culture.

NB2: Pools of 3 swabs can be performed but are not recommended for the diagnostic in case of clinical signs.

See § IV for the extraction and purification of RNA following the protocol specific to liquid biological samples.

B. Viral strain culture, supernatant of cellular culture, allantoidal liquids or bronchial liquids

Briefly centrifuge if necessary to clear bronchial liquids.

Take **200 µl of sample**.

NB: Store the rest of the liquid at -70°C +/- 10°C for a new analysis or for a viral culture.

See § IV for the extraction and purification of RNA following the protocol specific to liquid biological samples.

C. Tissue

RNA from tissue can be extracted according the dedicated protocol presented in following tables.

In case of plugging or inhibitions, RNA can also be extracted according to the protocol for liquid biological samples after the following treatment:

Put **0.1 g** of organ in a 2 ml-microtube with **1 ml** of MEM medium + antibiotic (in order to allow an ulterior viral culture) or of sterile saline water

Add **1 tungsten carbide or stainless-steel bead**.

Grind twice at 30 hertz for 3 minutes.

Take **200 µl of sample**.

NB: Store the rest of the liquid at -70°C +/- 10°C for a new analysis or for a viral culture.

See § IV for the extraction and purification of RNA following the protocol specific to liquid biological samples.

4. Controls to include

The use of controls allows verifying the reliability of the results.

The controls are included per trial of analysis. A trial is defined as all the samples treated in the same conditions.

All the steps of the analysis procedure (extraction+amplification), for all the types of samples, are validated with the association of the controls included in the kit.

- The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The SIV positive control allows validating the amplification of the target.

Other controls must or could be added:

- **Negative control of extraction (required)**

To verify the absence of cross-contamination, at least one negative control must be included per trial (e.g. the normative requirement and recommendation for the development and the validation of veterinary PCR NF U47-600 suggests the use of 1 negative control for 24 samples or 4 negative samples for a 96 wells-plate). This control could be a negative matrix, or a buffer used for dilutions.

- **Positive control of extraction (recommended)**

A positive control could be added in each trial. The control is a sample including SIV. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of SIV. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

V. Extraction and purification

1. Using RNeasy® kit

All the centrifugations are performed at room temperature.

	Liquid biological samples	Organs (lungs...)
Lysis	Add 350 µl of buffer RLT + β-mercaptoethanol (10µl/ml, optional) to the 200 µl of samples prepared as previously described. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Place 20-30 mg of the sample in a microtube. Add 350 µl of buffer RLT + β-mercapethanol (10µl/ml) and a metal bead . Grind 2 minutes at 30 Hz Briefly centrifuge the lysate. <i>If necessary, transfert the whole supernatant onto a QIAshredder spin column and centrifuge 2 minutes at 14 000 g.</i>
Binding preparation	Add 350 µl of ethanol 70% . Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply 700 µl of the obtained solution to the corresponding column. Centrifuge 1 minute at 8 000 g.	Identify columns, apply the whole obtained solution to the corresponding column. Centrifuge 1 minute at 8 000 g.
1 st wash	Change the collection tube and add 700 µl of buffer RW1 . Centrifuge 1 minute at 8 000 g.	
2 nd wash	Change the collection tube and add 500 µl of buffer RPE . Centrifuge 1 minutes at 8 000 g.	
3 rd wash	Change the collection tube and add 500 µl of buffer RPE . Centrifuge 3 minutes at 14 000 g.	
Elution	Transfer the column to a microtube. Add 50 µl of Nuclease-free water . Incubate ~2 minutes at room temperature and centrifuge 1 minute at 8 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

2. Using QIAamp® Viral RNA kit

All the centrifugations are performed at room temperature.

	Liquid biological samples	Organs (lungs...)
Lysis	Add 560 µl of buffer AVL + RNA carrier to the 200 µl of samples prepared as previously described. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Place 20-30 mg of the sample in a microtube. Add 560 µl of buffer AVL + RNA carrier and a metal bead. Grind 2 minutes at 30 Hz
	Incubate at room temperature during 10 minutes. Briefly centrifuge the lysate.	
Binding preparation	Add 560 µl of ethanol 100%. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply 630 µl of the obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. Change the collection tube, put the rest of the mix on the column and centrifuge 1 minute at 10 000 g.	
1 st wash	Change the collection tube and add 500 µl of buffer AW1. Centrifuge 1 minute at 10 000 g.	
2 nd wash	Change the collection tube and add 500 µl of buffer AW2. Centrifuge 1 minutes at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	Transfer the column to a microtube. Add 60 µl of buffer AVE. Incubate ~2 minutes at room temperature and centrifuge 1 minute at 10 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

3. Using Nucleospin® RNA kit

All the centrifugations are performed at room temperature.

	Liquid biological samples	Organs (lungs...)
Lysis	Add 350 µl of RA1 buffer + β-mercaptoethanol (10µl/ml, optional) to the 200 µl of samples prepared as previously described. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Place 20-30 mg of the sample in a microtube. Add 350 µl of RA1 buffer + β-mercapethanol (10µl/ml) and a metal bead. Grind 2 minutes at 30 Hz Briefly centrifuge the lysate. <i>If necessary, transfert the whole supernatant onto a filtration column and centrifuge 1 minute at 11 000 g.</i>
Binding preparation	Add 350 µl of ethanol 70%. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply 700 µl of the obtained solution to the corresponding column. Centrifuge 30 seconds at 11 000 g.	Identify columns, apply the whole obtained solution to the corresponding column. Centrifuge 30 seconds at 11 000 g.
1 st wash	Change the collection tube and add 350 µl of buffer MDB. Centrifuge 1 minute at 11 000 g.	
2 nd wash	Add 200 µl of buffer RAW2. Centrifuge 30 seconds at 11 000 g.	
3 rd wash	Change the collection tube and add 600 µl of buffer RA3. Centrifuge 30 seconds at 11 000 g.	
4 th wash	Change the collection tube and add 250 µl of buffer RA3. Centrifuge 2minutes at 11 000 g.	
Elution	Transfer the column to a microtube. Add 60 µl of Nuclease-free water. Incubate ~2 minutes at room temperature and centrifuge 1 minute at 11 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

4. Using Nucleospin® RNA Virus kit

All the centrifugations are performed at room temperature.

	Liquid biological samples	Organs (lungs...)
Lysis	Add 560 µl of buffer RAV1 + carrier pre-warmed at +56°C to the 200 µl of samples prepared as previously described. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Place 20-30 mg of the sample in a microtube. Add 560 µl of buffer RAV1 + carrier pre-warmed at +56°C and a metal bead. Grind 2 minutes at 30 Hz
	Incubate at room temperature during 10 minutes. Briefly centrifuge the lysate.	
Binding preparation	Add 560 µl of ethanol 100%. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply 630 µl of the obtained solution to the corresponding column. Centrifuge 1 minute at 8 000 g. Change the collection tube, put the rest of the mix on the column and centrifuge 1 minute at 8 000 g.	
1 st wash	Change the collection tube and add 500 µl of buffer RAW. Centrifuge 1 minute at 8 000 g.	
2 nd wash	Change the collection tube and add 600 µl of buffer RAV3. Centrifuge 1 minutes at 8 000 g.	
Column dry step	Change the collection tube. Centrifuge 5 minutes at 11 000 g.	
Elution	Transfer the column to a microtube. Add 50 µl of Nuclease-free water. Incubate ~2 minutes at room temperature and centrifuge 1 minute at 11 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

5. Using Nucleospin® 8 RNA kit

All the centrifugations are performed at room temperature.

	Liquid biological samples	Organs (lungs...)
Lysis	Add 350 µl of buffer RA1 + β-mercaptoethanol (10µl/ml, optional) to the 200 µl of samples prepared as previously described. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	Place 20-30 mg of the sample in a microtube. Add 350 µl of buffer RA1 + β-mercapethanol (10µl/ml) and a metal bead. Grind 2 minutes at 30 Hz Briefly centrifuge the lysate. <i>If necessary, transfert the whole supernatant onto a filtration strip and centrifuge 1 minute at 6 000 g.</i>
Binding preparation	Add 300 µl of buffer RA4 previously completed with ethanol 100%. Homogenize by pipetting (~10 times) or by using a mixer such as vortex (~15 seconds).	
Transfer to columns and binding to the membrane	Identify columns, apply 700 µl of the obtained solution to the corresponding well of the blue strip. Centrifuge 2 minutes at 6 000 g.	Identify columns, apply the whole obtained solution of the obtained solution to the corresponding well of the blue strip. Centrifuge 2 minutes at 6 000 g.
1 st wash	Transfer strips on non-used trash wells or change the collection plate. Add 500 µl of buffer RA3. Centrifuge 2 minutes at 6 000 g.	
2 nd wash	Transfer strips on non-used trash wells or change the collection plate. Add 500 µl of buffer RA2. Centrifuge 2 minutes at 6 000 g.	
3 rd wash	Transfer strips on non-used trash wells or change the collection plate. Add 800 µl of buffer RA3. Centrifuge 2 minutes at 6 000 g.	
4 th wash	Transfer strips on non-used trash wells or change the collection plate. Add 500 µl of buffer RA4. Centrifuge 10 minutes at 6 000 g.	
Elution	Transfer the strips on elution tube strips. Add 75 µl of Nuclease-free water. Incubate ~2 minutes at room temperature and centrifuge 3 minutes at 6 000 g.	
Storage	Close the tubes, identify and store on ice if using immediately or at <-15°C.	

6. Using Nucleospin® 96 Virus

Three MN Square well block plates are included in each kit. They are used as mix plates or recovery plates. Once they have been used, they can be emptied, decontaminated with HCl 0.4 M during 1 minute, washed with distilled water and autoclaved.

All centrifugations are achieved at 5900 tr/min (5600 to 5800 g) and at room temperature.

Before the beginning of extraction, pre-warm:

- the RAV1 buffer + RNA carrier at +56°C.
- the Nuclease-free water at +70°C.

	Liquid biological samples
Lysis	Place 200 µl of sample prepared as previously described in each well of a Round-well Block plate.
	Add 560 µl of buffer RAV1 + RNA carrier pre-warmed at +56°C + 20 µl of proteinase K . Homogenize the mix 5-times with a multichannel pipette P1000. Close the plate with an adhesive seal Self-adhering PE Foil. Incubate 10 minutes at +70°C.
Binding preparation	Place 560 µl of ethanol 100 % in an MN Square well Block plate. Carefully remove the adhesive seal of the Round-well Block plate containing the samples and transfer the whole content of in the MN Square well Block plate containing ethanol. Homogenize the mix 10-times (very important) with a multichannel pipette P1000.
Transfer to columns and binding to the membrane	Place a Nucleospin® Virus Binding plate (blue) on a new MN Square well Block plate. Transfert the whole mix with a multi pipette P1000 on the Nucleospin® Virus Binding plate. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 2 minutes. If the whole mix has not filtered, centrifuge one more time 3 minutes.
1 st wash	Place the Nucleospin® Virus Binding plate on a new MN Square well Block plate. Remove the adhesive seal from the Nucleospin® Virus Binding plate. Add 500 µl of buffer RAW in each well. Place a new adhesive seal Self adhering PE foil on the plate. Centrifuge 2 minutes.
2 nd wash	Remove the adhesive seal of the Nucleospin® Virus Binding Plate. Add 900 µl of buffer RAV3 in each well. Place a new adhesive seal Self adhering PE Foil on the plate. Centrifuge 5 minutes.
Column dry step	Place the Nucleospin® Virus Binding Plate on an empty and dry 96 well plate (ELISA-like). Centrifuge 10 minutes.
Elution	Place the Nucleospin® Virus Binding Plate on the Rack plate with MN tube strips. Remove the adhesive seal from the plate. Add 100 µl of Nuclease-free water pre-warmed at +70°C in each well of the Nucleospin® Virus Binding plate. <u>Do not use the buffer RE.</u> Incubate ~2 minutes at room temperature and centrifuge 2 minutes.
Storage	Remove the Nucleospin® Virus Binding plate. Close the Rack plate with MN tube strips with Caps for strips. Store it on melting ice if analysis is immediately achieved, then at <-15°C.

7. Using ADIAMAG kit

See the NEKF user manual available of the web site mentioned on the certificate of analysis included in the used ADIAVET™ kit.

VI. Amplification

a - Determine the number samples analysed including the controls (e.g. positive and negative extraction controls, control of amplification (positive and negative controls) and PCR reagent control (NTC)).

b - Defrost the A5 solution at room temperature. Homogenize. Dispense **20 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace the A5 solution tube at <-15°C and in darkness.**

d- For each sample, the extraction negative control (obligatory) and the extraction positive control (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.

For the controls, add **5 µl**, per well, of each solution obtained in § II-3 to the 20 µl of A5 solution. For the PCR reagent control (NTC), nothing is added to the A5 solution.

Immediately replace purified RNA extracts on melting ice or at <-15°C. Take care to have no bubbles in the bottom of the wells.

e- Store the plate or the tubes on melting ice or at +2/8°C until the cyclor is programmed and start quickly the run after you have placed the plate or the tubes in the cyclor.

The A/H1N1 target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step (1 minute at 60°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:

10 minutes 45°C

10 minutes 95°C

15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:

10 minutes 45°C

10 minutes 95°C

30 seconds at 95°C and 1 minute at 60°C during 45 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*

*** NOTE:** *The use of LightCycler thermalcyclers requires a calibration manipulation. Adiogene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

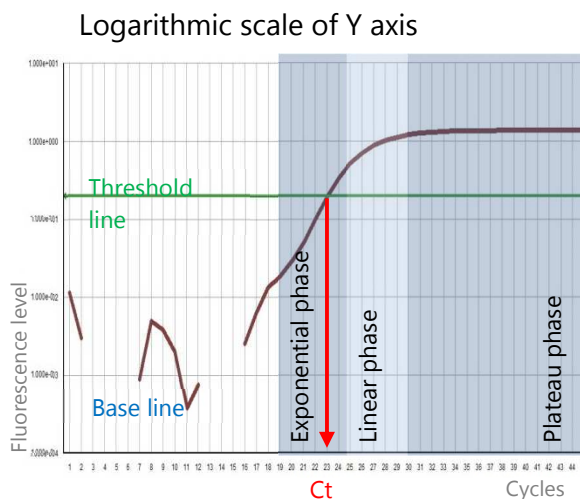
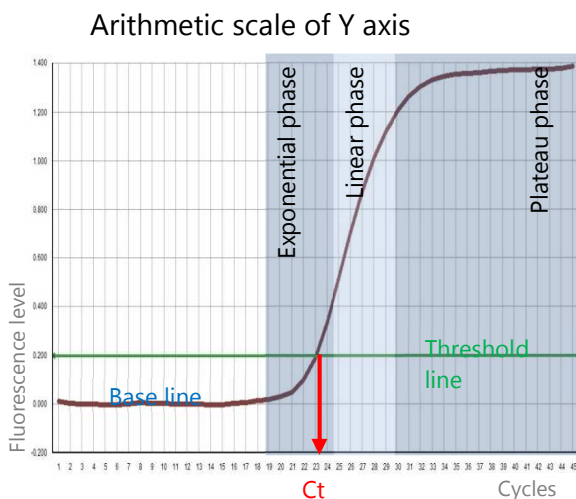
The « **base line** » corresponds to the background of fluorescence and qualifies the non characteristic part of the curve observed during the first cycles.

The « **Characteristic amplification curve** » qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The « **threshold line** » has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

The « **threshold cycle** » (**Ct**) of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve



2. Validation and interpretation of results

*Display the FAM curves from the plate and set the threshold value as indicated above.
Proceed in the same mean for the VIC or HEX curves.*

A. Validation of the run

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	A/H1N1 CTL+	SIV CTL-	Extraction negative control	Extraction positive control *
FAM amplification	no	Yes	no	no	yes
VIC/HEX amplification	no	no/yes	yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the A/H1N1 target	Amplification of the IPC target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the A/H1N1 CTL+ were indicated in the certificate of analysis of the kit.

B. Result interpretation

RNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for A/H1N1 (FAM) or for the internal control (VIC or HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (A example).

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (B example). Internal control can be co-amplified (C example).

A total absence of characteristic amplification curve for a sample (example D) shows a defective RNA extraction (lost or destruction of RNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted RNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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