Use of MACHEREY-NAGEL´s NucleoMag™ 96 Virus kit for the detection of influenza virus A/H1N1

Abstract

The 2009 flu pandemic is a global outbreak of a new strain of influenza A virus subtype H1N1. The outbreak was first observed in Mexico, with evidence that there had been an ongoing epidemic for months before it was officially recognized as such. In the present study we describe the automated use of the MACHEREY-NAGEL NucleoMag™ 96 Virus kit on the KingFisher® Flex magnetic separator for the isolation of viral nucleic acids and subsequent detection by PCR. For kit evaluation A/H1N1 samples from an interlaboratory test as well as clinical samples have been used.

Introduction:

The so called „swine flue" is caused by the swine influenza virus (A/H1N1). This virus was discovered first in 1930 in pig populations. The recently discovered subtype A of the H1N1 virus includes genes derived by reassortment from human, swine and avian viruses, which can be transmitted from human to human. As for all influenza caused infections a reliable and sensitive detection of the virus is an important prerequisite to identify suspicious patients and to support infection control.

For the detection of influenza viruses rapid tests using antibodies to detect virus proteins are available and commonly used. However, tests are often less sensitive in comparison to real-time RT-PCR based methods and prone to false negative results. Therefore the RT-PCR based methods are considered as the gold standard.

Typically swabs or nasopharyngeal swab samples are taken from patients and used for viral RNA extraction. There are high demands for RNA extraction methods with regard to sensitivity and reproducibility. Moreover, since usually a high number of samples hase to be screened the extraction is typically automated with a high throughput.

In the current application note we describe the use of the MACHEREY-NAGEL magnetic beads based NucleoMag™ 96 Virus kit in combination with the Thermo KingFisher® Flex magnetic particle separator. Swabs are washed and incubated for example in NaCl solution. The method starts with a lysis incubation of the swab wash solution and subsequently, after addition of binding buffer, the released nucleic acids are bound to magnetic particles. Following several washing steps the purified viral RNA is eluted and can be used for RT-PCR down stream applications. The use of the KingFisher® Flex separation device allows the purification of up to 96 samples within approx 60 min.

Material and Methods:

The kit evaluation was done by Labcon OWL, Bad Salzuflen, Germany, with clinical samples and INSTAND interlaboratory test samples.

Samples that have been used in the study comprise

- INSTAND round robin samples for influenza A/H1N1. Samples are suitable for the detection of human influenza A and B viruses as well as new influenza A subtype H1N1 (swine flue).
- Samples are provided as lyophilized lysates of tissue culture media or allantoic liquid from infected and incubated hen´s eggs.
- Samples have been extracted with the NucleoMag™ 96 Virus kit automated on a KingFisher® Flex magnetic separator. After elution aliquots of the eluates have been used in conjunction with two different PCR system for the detection of influenza A and A/H1N1.

In addition to the samples from the interlaboratory test swab samples from potentially positive patients were subjected for viral RNA isolation.

The purification protocol used is summarized in Table 1.

Table 1: Purification protocol for viral RNA isolation using the NucleoMag™ 96 Virus kit on the KingFisher® Flex instrument.

Plate	KingFisher® Flex 96
Plate 1 (Thermo KF 96 plate)	KF 96 Plate and KF 96 Tip comb
Plate 2 (Thermo deep-well plate)	200 µl sample, 200 µl Lysis buffer MV1, 10 µl Proteinase K, 4 µl carrier RNA Mixing 15 min, fast, 56 °C Add 600 µl Binding buffer MV2 and 30 µl V-beads Mixing: 5 min, bottom mix, collect 3x
Plate 3 (Thermo deep-well plate)	Wash buffer MV3, 500 µl Mixing: 2 min, half mix, collect 3x
Plate 4 (Thermo deep-well plate)	Wash buffer MV4, 500 µl Mixing: 2 min, half mix, collect 3x
Plate 5 (Thermo deep-well plate)	Wash buffer MV5, 550 µl 90 sec, slow, beads are not re-suspended in Wash buffer MB5
Plate 5 (Thermo deep-well plate)	Air-drying step, 3 min
Plate 6 (Thermo KF 96 plate)	Elution buffer MB6, 70 µl heating (preheat) 56 °C, Mixing: 5 min, slow, collect 3x release beads into plate 4

For downstream PCR analysis two different PCR systems have been used:

Real-time RT-PCR amplification was performed according to the recommendations of the Robert Koch-Institute (RKI) or using a commercial detection kit (Astra Diagnostics, Germany).

Results

1) Method development using influenza INSTAND interlaboratory test samples

RT-PCR according to RKI recommendations (Lightcycler® 480)

This RT-PCR system uses two independent master mixes. One RT-PCR reaction detects influenza A viruses, while the second PCR reaction is specific for subtype H1N1. Consequently, a sample which is positive for H1N1 should be positive in both PCR reactions. Influenza A subtypes different from H1N1 should yield only positive signals in the influenza A reaction.

The results are summarized in Table 2.

Table 2: Results from INSTAND interlaboratory test samples. Real-time PCR according to the recommendations of the RKI.

RR-no.	sample specification	In House PCR (RKI)	
		Influenza A	Influenza H1N1
20088	positive for influenza B (seasonal)	negative	negative
20089	positive for influenza A/H1N1 (swine lineage)	positive	positive
20090	positive for influenza A/H3N2 (seasonal)	positive	negative
20091	negative for influenza A and B	negative	negative
20092	positive for avian influenza A/H5N1 (seasonal)	positive	negative
20093	positive for influenza A/H1N1 (seasonal)	positive	negative
20094	positive for influenza A/H1N1 (swine lineage)	positive	positive
20095	positive for avian influenza A/H5N1	positive	negative

RT-PCR kit from Astra Diagnostics, Hamburg, Germany (Influenza Screen & Type RT-PCR Kit 1.0)

This PCR system uses a mastermix for the detection of both influenza A and subtype H1N1 (the primers bind to similar target sequences). Only one of the primers (either influenza A or H1N1) bind to the target and only one signal is generated: one PCR reaction detects influenza A viruses, while the second PCR reaction is specific for subtype H1N1. Consequently, a sample which is positive for H1N1 should be negative for influenza A. Influenza A subtypes different from H1N1 should yield only positive signals in the influenza A reaction.

The results are summarized in Table 3.

Table 3: Results from INSTAND interlaboratory test samples. Real-time RT-PCR kit from Astra Diagnostics.

RR-no.	sample specification	Astra Kit	
		Influenza A	Influenza H1N1
20088	positive for influenza B (saisonal)	negative	negative
20089	positive for influenza A/H1N1 (swine lineage)	negative	positive
20090	positiv for influenza A/H3N2 (saisonal)	positive	negative
20091	negative for influenza A and B	negative	negative
20092	positive for avian influenza A/H5N1 (saisonal)	positive	negative
20093	positive for influenza A/H1N1 (saisonal)	positive	negative
20094	positive for influenza A/H1N1 (swine lineage)	negative	positive
20095	positive for avian influenza A/H5N1	positive	negative

As can be seen from Table 2 and 3 with both PCR systems all samples from the interlaboratory test have been identified correctly. Neither false positives nor false negatives have been observed.

2) Testing of the method with swab samples from patients

The isolated viral RNA from swab samples was subjected to RT-PCR reactions specific for either influenza A or H1N1. RT-PCR setup was performed according to the recommendations of the Robert Koch-Institute (RKI) on a Roche LightCycler® 480 instrument. Amplification plots are shown in Figure 1 (influenza A) and Figure 2 (H1N1). Calculated CP values are summarized in Figure 3

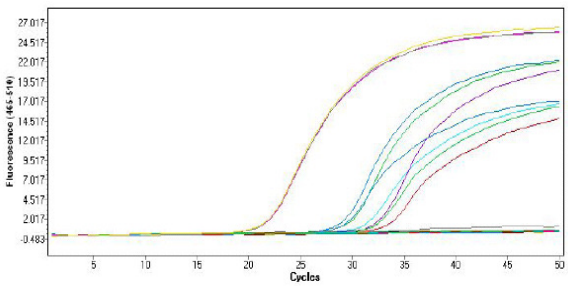

Figure 1: Amplification plots for influenza A specific RT-PCR.

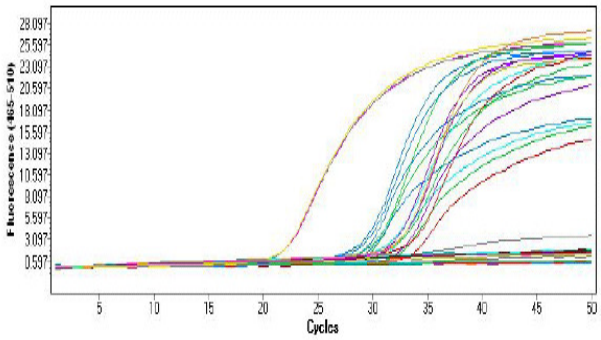

Figure 2: Amplification plots for H1N1 specific RT-PCR.

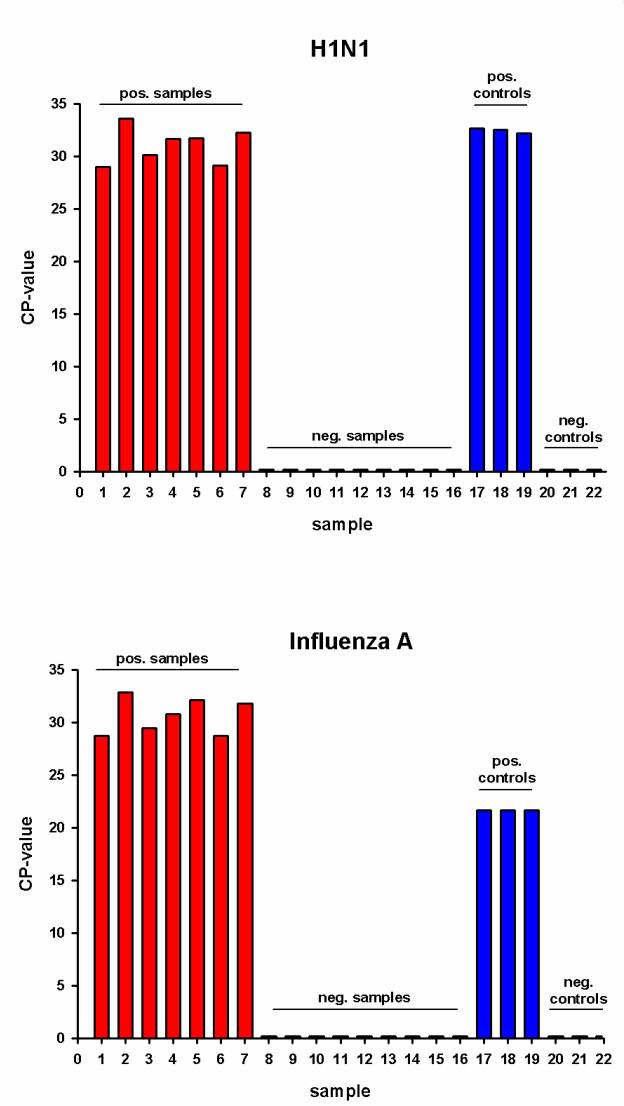

Figure 3: Summary for RT-PCR detection of H1N1 (upper figure) and influenza A (lower figure). Amplification controls: black bars: negative controls; blue bars: positive controls.

As can be seen in Fig. 3 all samples which were tested positive for the presence of the H1N1 virus (Fig. 3, top) were also tested positive for influenza A (Fig. 3, bottom), clearly identifying the samples

positive for the swine virus A/H1N1. All negative H1N1 also proved to be negative for influenza A. In both RT-PCR systems the negative controls were always negative whereas positive controls were positive.

Summary

With both RT-PCR systems all samples from the interlaboratory test have been identified correctly. Neither false positives nor false negatives have been observed. For swab samples consistent results were obtained. This supports the excellent quality of the eluted viral RNA and the unlimited use for downstream RT-PCR.

Acknowledgement

We are grateful to Dr. Carsten Tiemann, Mrs Andrea Hartmann and Mrs Daniela Knoop at Labcon OWL in Bad Salzuflen for providing clinical and interlaboratory test samples and for conducting PCR analysis.

References:

Robert-Koch Institut, www.rki.de. TaqMan® real-time PCR for detection of porcine influenza A/H1N1-virus

MACHEREY-NAGEL:
MACHEREY-NAGEL GmbH & Co. KG, Neumann-Neander-Str. 6-8,. D-52355 Dueren, Germany
Tel +49 (0) 24 21 -270, Fax +49 (0) 24 21 969-199, E-mail: tech-bio@mn-net.com, *www.mn-net.com*

Ordering Information:

Product	Preps	Cat. No.
NucleoMag™ 96 Virus	1 x 96	744 800.1
NucleoMag™ 96 Virus	4 x 96	744 800.4
KingFisher® 96 Accessory Kit A	1 set	744 950

For more information regarding the automated use of MN products, please contact your local representative