INSTRUCTION MANUAL

ADIAVET™ M.HYOP REAL TIME

TEST FOR THE DETECTION OF *MYCOPLASMA HYOPNEUMONIAE* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
418022 (100 reactions)

VET

NOTE
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English version
NE054-05
2020/01

ADIAVET™ M.HYOP REAL TIME

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
2014/02	NE054-02	Technical change	Addition of reference 418022 (100 reactions)
2014/12	NE054-03	Technical change	Removal of reference 416441 (50 reactions)
2016/07	NE054-04	Administrative	Changing logos
2016/07	NE054-04	Administrative	Biosearch legal mention
2016/07	NE054-04	Administrative	Addition of table "Analysis options according to the specimen"
2016/07	NE054-04	Correction	Modification of NucleoSpin Tissue protocol (§IV.2.)
2020/01	NE054-05	Technical change	Addition of a NF-Water tube in the kit Modification of "RNA/DNA extraction kit" by "ADIAMAG" kit

II. General information

1. Purpose of the test

ADIAVET™ M.HYOP REAL TIME kit is intended to detect *Mycoplasma hyopneumoniae* using real-time Polymerase Chain Reaction (PCR) technology from tissue, tracheobronchiolar washing and oral fluid specimens of pig, as well as from bacterial culture.

2. Pathogen

Mycoplasma hyopneumoniae is the primary agent of enzootic porcine pneumonia (EPP), one of the most important direct or indirect causes of respiratory infectious diseases. The disease has a world wide distribution and causes considerable economic losses in swine production due to reduce growth rate and feed conversion efficiency. *M. hyopneumoniae* has a specific pathogenicity and leads to secondary infection by other pathogenic bacteria such as *Pasteurella multocida* or *Actinobacillus pleuropneumoniae*. The contamination occurs at each stage of piglet production, from sows to piglets but also from pigs themselves by direct contact (Kobisch and Friis, 1996).

Diagnosis:

The isolation of *M. hyopneumoniae* by culture is not currently performed by diagnostic laboratories because it is tedious and time consuming and may require as long as 1 month (Friis, 1975). Moreover, in the respiratory tract of piglets it is often associated with bacteria (*P. multocida*, *A. pleuropneumoniae*) as well as other mycoplasma species (*M. hyorhinis* and *M. flocculare*). Two methods are currently used for EPP diagnosis: serological analysis such as blocking ELISA or detection of the organism on lung sections by a direct immunofluorescent test using polyclonal antibodies (L'Ecuyer et Boulanger, 1970). Many authors have described *M. hyopneumoniae* detection by PCR test. This technique allowed the specific and rapid detection of mycoplasma directly on live pigs (Mattsson et al., 1995; Verdin et al ., 1996; Baumeister et al., 1998). The PCR test allowed the detection of *M. hyopneumoniae* on seropositive pigs (infected or vaccinated pigs).

3. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ M.HYOP REAL TIME kit enables the simultaneous detection of:

- *Mycoplasma hyopneumoniae* with probe labelled in FAM.
- The RNase P, an internal control of extraction and amplification steps specific from an endogenous DNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE recommends using this test with DNA purification kits (Bio-X Diagnostics, Qiagen or 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
Tissue (lung)	☑
Tracheobronchiolar washing	☑
Oral fluid	☑
Bacterial culture	☑

III. Material and reagents

1. Reagents provided with the kit

REF 418022 (100R)		
A5	amplification solution	2 x 1000 µl tube with green cap (a ready-to-use reagent)
M.HYOP CTL+	positive control *Mycoplasma hyopneumoniae*	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)

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 M.HYOP CTL+

Add **200 µl** of **NF-Water** to the **M.HYOP CTL+** tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use **5 µl** of **M.HYOP 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
- Etuve, heating baths or block heaters
- Instrument for homogenous mixing of tubes
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Powder-free latex or nitrile gloves
- 96-100% ethanol solution
- PBS 1X pH7.4 buffer

- DNA extraction kit (individual columns)

- QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)
- NucleoSpin® Tissue (Macherey-Nagel, 50 tests: ref.740952.50 or 250 tests: ref. 740952.250)

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

ADIAGENE has elaborated this PCR test with the use of Bio-X Diagnostics, Qiagen and Macherey-Nagel extraction kits. 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 a 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 25 minutes at +120°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 DNAs are quite sensitive molecules. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for 24h hours, then at <-15°C.

3. Samples preparation

See § IV for the extraction and purification of DNA.

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.
The use of the controls follows the recommendation of the the normative requirement and recommendation for the development and the validation of veterinary PCR (NF U47-600).

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 (RNase P) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The M.HYOP CTL+ allows validating the amplification of the target.

Other controls must or could be added.

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

  To verify the absence of cross-contamination, at least one negative control must be included per trial. The control is a negative sample, for example 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 *M. hyopneumoniae* target. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *M. hyopneumoniae*. 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 QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.
Before the beginning of the extraction, turn on the heating system at +70°C.

	Lung	**Tracheobronchiolar washings**	**Oral fluid** **Bacterial culture**
Preparation of the sample	Take **20 mg** of lung in microtube **or** rub the bronchiole lung with a swab and introduce the swab in microtube (cut the stem if necessary).	Place **1 ml** of tracheobronchiolar washing in a microtube. Centrifuge 30 minutes at 10 000g. Discart the supernatant.	Place **200 µl** of sample in a microtube.
Lysis	Add **180 µl** of **ATL buffer, 20 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C**.		
	Add **200 µl** of **AL buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).		
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. *If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*		
1st wash	Change the collection tube and add **500 µl** of **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.		
2nd wash	Change the collection tube and add **500 µl** of **AW2 buffer** to the column. Centrifuge 1 minute 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 **200 µl** of **AE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.		
Storage	Close the tubes, identify and store at +2/8°C for 24h hours, then at <-15°C.		

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.
Before the beginning of the extraction, turn on the heating system at +70°C.

	Lung	**Tracheobronchiolar washings**	**Oral fluid** **Bacterial culture**
Preparation of the sample	Take **20 mg** of lung in microtube **or** rub the bronchiole lung with a swab and introduce the swab in microtube (cut the stem if necessary).	Place **1 ml** of tracheobronchiolar washing in a microtube. Centrifuge 30 minutes at 10 000g. Discart the supernatant.	Place **200 µl** of sample in a microtube.
Lysis	Add **180 µl** of **T1 buffer, 25 µl** of **proteinase K**. Homogenize. Incubate **30 minutes** at **+70°C**.		
	Add **200 µl** of **B3 buffer**. Homogenize. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 seconds).		
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. *If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*		
1st wash	Change the collection tube and add **500 µl** of **BW buffer** to the column. Centrifuge 1 minute at 10 000 g.		
2nd wash	Change the collection tube and add **600 µl** of **B5 buffer** to the column. Centrifuge 1 minute 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 **200 µl** of **BE buffer**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.		
Storage	Close the tubes, identify and store at +2/8°C for 24h hours, then at <-15°C.		

3. Using ADIAMAG kit

See the NEKF user manual available on 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, positive control of amplification (CTL+) and reagent PCR control (NTC).

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

c- **Immediately replace the A5 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 the A5 solution.
For the CTL+, add **5 µl** of the 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 DNA extracts at +2/8°C or at <**-15°C**. Take care to have no bubbles in the bottom of the wells.

e- Once all the tubes have been prepared, run real-time PCR amplification.

The *Mycoplasma hyopneumoniae* 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**:
2 minutes 50°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**:
2 minutes 50°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. Adiagene 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.

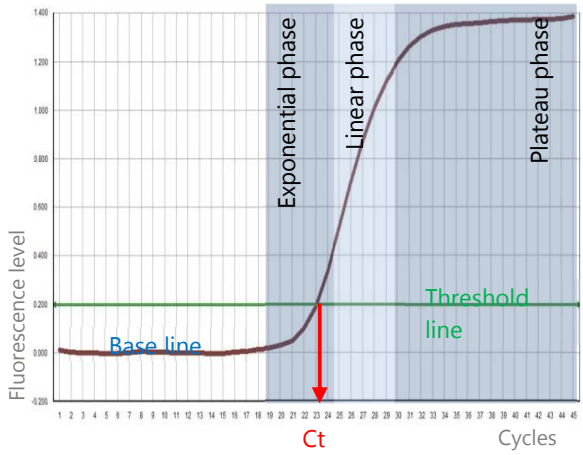
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

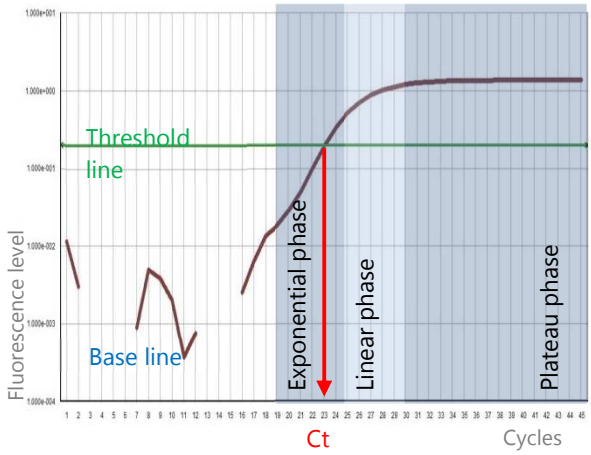
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



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	No Template Control (NTC)	M.HYOP CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	no	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the M.HYOP CTL+ are indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for *Mycoplasma hyopneumoniae* (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 DNA extraction (lost or destruction of DNA) 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 DNA in Nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. References:

Baumeister A. K., Runge M., Ganter M., Feenstra A. A., Delbeck F. and Kirchoff H. (1998). Detection of *M. hyopneumoniae* in bronchoalveolar lavage fluids of pigs by PCR. J. Clin. Microbiol. 36: 1984-1988.

Friis N. F. (1975). Some recommendations concerning primary isolation of *M. suis, M. Hyopneumoniae* and *M. flocculare*. A survey. Nord. Vet. Med. 27: 337-339.

Kobisch M and Friis. N.F. (1996). Swine mycoplasmoses. Rev. Sci. Tech. Off. Epiz. 15(4): 1569-1605.

L'Ecuyer C. & Boulanger P. (1970). Enzootic pneumonia of pigs: identification of a causative mycoplasma in infected pigs and in cultures by immunofluorescent staining. Can. Comp. Med. 34: 38-
46.

Mattsson J. G., Bergstöm K., Wallgren P. and Johansson K. E. (1995). Detection of *Mycoplasma hyopneumoniae* in nose swabs from pigs by in vitro amplification of the 16S RNA gene. J.Clin. Microbiol. 33: 893-897.

Verdin E., Blanchard B., Kobisch M., Bove J. M. and Saillard C. (1996) Use of a nested PCR diagnosis test to detect *M. hyopneumoniae* under field conditions. IOM lett. 4: 101-102.

IX. 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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