INSTRUCTION MANUAL

ADIAVET™ MYCO AV REAL TIME

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

Reference:
417996 (100 reactions)

VET

NOTE
Dye compounds in this product are sold under license from Biosearch Technologies, Inc. and protected by U.S. and worldwide patents either issued or in application. The license covers Veterinary Molecular Diagnostics.

English version
NE015-03
2020/01

ADIAVET™ MYCO AV REAL TIME

Revision historic

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

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

Release Date	Part Number	Change type	Change summaru
2014/09	NE015-01	N/A	First publication
2016/07	NE015-02	Administrative	Changing logos
2016/07	NE015-02	Administrative	Biosearch legal mention
2016/07	NE015-02	Administrative	Addition information on the detection of vaccines Addition of table "Analysis options according to the specimen"
2016/07	NE015-02	Correction	Modification of NucleoSpin Tissue protocol (§IV.2.)
2020/01	NE015-03	Technical change	Addition of a NF-Water tube in the kit ADIAPURE™ PURIFICATION protocol suppressed Suppression of 500 reaction reference

I. General informations

1. Purpose of the test

ADIAVET™ MYCO AV REAL TIME kit is intended to detect *Mycoplasma synoviae* and *Mycoplasma gallisepticum* using real-time Polymerase Chain Reaction (PCR) technology from swab of poultry, environmental specimen and bacterial culture.

2. Avian mycoplasms

Mycoplasma are known to cause economic losses in commercial poultry production. *M. gallisepticum* causes chronic respiratory disease in chickens and infection sinusitis in turkeys. *M. synoviae* most frequently occurs as subclinical upper respiratory infection and synovitis in chickens and turkeys.
Rapid identification of any of these mycoplasma organisms is of great importance to the poultry industry. The diagnosis of avian mycoplasma infections is mainly based on culture and serology (RSA, ELISA...) (Stipkovitch & Kempf, 1996). However culture is rather slow, tedious, expensive and often contaminated.
Serological tests are useful but sometimes lack specificity or sensitivity (Yamamoto, 1991). Recently PCR tests have been developed providing specific and sensitive tools for avian mycoplasma detection (Kempf, 1997).

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™ MYCO AV REALTIME kit enables the simultaneous detection of:

- *M. synoviae* (probe labelled in FAM),
- *M. gallisepticum* (probe labelled in Cy5),
- an internal control of amplification step specific from an exogenous DNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

The test detects the MG TS-11 and MS-H vaccine strains but not the MG 6/85 and MG-F vaccine strains.

Adiagene recommends using this test with DNA purification kits (Qiagen and 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
Swab on live animals (palate slit, tracheal…)	☑	6
Swab on dead animals (joint, injured organ…)	☑	6
Environmental specimen	☑	☒
Bacterial culture (solid, liquid)	☑	☒

* It depends on the epidemiological case and on the quality of the specimen.

II. Material & reagents

1. Reagents provided with the kit

REF 417996 (100R)		
A5	Amplification Solution	2 x 1000 µl tube green caps (ready to use)
MS CTL+	Positive control *M. synoviae*	1 tube purple caps (dehydrated)
MG CTL+	Positive control *M. gallisepticum*	1 tube purple caps (dehydrated))
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 MS CTL+

MS CTL+ is a positive control of amplification.

Add **200 µl** of **NF-Water** to the **MS CTL+** tube and vortex at least 20 seconds.

Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, use **5 µl** of **MS CTL+** in one of the wells.

4. Use of MG CTL+

MG CTL+ is a positive control of amplification.

Add **200 µl** of **NF-Water** to the **MG CTL+** tube and mix by vortexing at least 20 seconds.

Aliquot this solution by 6 or 12 µl and store them to <-15°C.

For each analysis, use **5 µl** of **MG CTL+** in one of the wells.

5. Equipment required but not supplied

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
- Vortex
- 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
- Sterile tube of 5, 10 or 15 ml
- Latex or nitrile powder-free gloves
- 96-100% ethanol solution
- Peptone water
- Sterile saline water (NaCl 8.5 g/l)
- **DNA extraction kit (individual silica 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)

III. Recommendations 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 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 handled 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 the samples to be tested, and another one for the 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 24 hours, then at <-15°C.

3. Samples preparation

A. From swabs

In case of very soiled swabs, prefer individual analysis.

1st method

Put **1 swab** or vortex successively **up to 3 swabs** in a microtube containing **the first lysis buffer of the extraction kit used** (see § IV.).
NB: if the swabs haven't been dipped, they may absorb all the solution. In this case, the amount of lysis buffer should be doubled.
Mix.
Press the swab against the rim of the microtube to collect as much buffer as possible.
See § IV for the extraction and purification of DNA.

2nd method

Cut **1** to **3 swabs** in a tube (e.g. a 5 ml – tube) containing **700 µl** of **physiological water**.
Vortex ~10 seconds.
Transfer **200 µl** of **supernatant** in a microtube.
See § IV for the extraction and purification of DNA.
NB: swabs in physiological water can be stored at +2/8°C.

B. From solid culture

Scrape the medium with a swab (dipped in 2% peptone water).
Put it in a microtube containing **the first lysis buffer of the extraction kit used** (see § IV.).
Vortexing ~5 seconds.
Press the swab against the rim of the microtube to collect as much buffer as possible.
See § IV for the extraction and purification of DNA.

C. From liquid culture

Centrifuge (20 minutes at 10 000 g) **100** to **500 µl** of **culture** in a microtube.
Discard the supernatant.
See § IV for the extraction and purification of DNA.

D. From drag swabs

Add **30 ml** of **peptone water** in the bag containing the drag swab.
Homogenize by mixing the bag.
NB: If the drag swab has absorbed all the liquid, add 10 ml more peptone water and mix the bag again.
Transfer **200 µl** of **supernatant** in a microtube.
See § IV for the extraction and purification of DNA.

4. Controls preparation

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 amplification step, for all the types of samples, is validated with the association of the controls included in the kit.

- The internal control included in A5 reagent verifies the amplification steps of each sample.
- The CTL+ validate the amplification of the both 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. 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. synoviae* and/or *M. gallisepticum*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *M. synoviae* and/or *M. gallisepticum*. 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.

IV. Extraction and Purification

1. Using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

	Swab	Bacterial culture	Drag swabs
Preparation of the sample	See § III.3.		
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Vortex. Incubate **15 minutes** at **+56°C**.		
	Add **200 µl** of **AL buffer**. Vortex. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortex (~15 secondes).		
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 24 hours, then at <-15°C.		

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	Swab	Bacterial culture	Drag swabs
Preparation of the sample	See § III.3.		
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Vortex. Incubate **15 minutes** at **+56°C**.		
	Add **200 µl** of **B3 buffer**. Vortex. Incubate **10 minutes** at **+70°C**.		
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortexing (~15 secondes).		
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge at 10 000 g/1 minute. *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 24 hours, then at <-15°C.		

V. Amplification

a - Determine the number of analysed samples including the controls (e.g. positive and negative extraction controls, positive control of amplification (CTL+) and No Template Control (NTC)).

b - Defrost the A5 solution at room temperature. Vortex. 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 A5 solution.
For the CTL+, add **5 µl** of the solution obtained in § II-3 or § II-4 to the 20 µl of A5 solution.
For the No Template 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 the real-time PCR amplification.

The *M. synoviae* target is read in FAM. The *M. gallisepticum* target is read in Cy5. 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.

VI. 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.

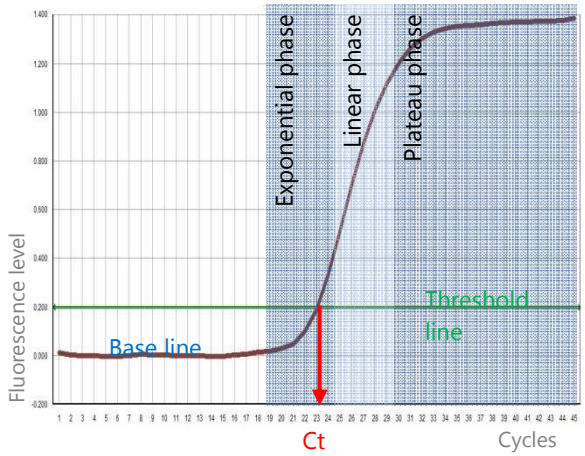
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

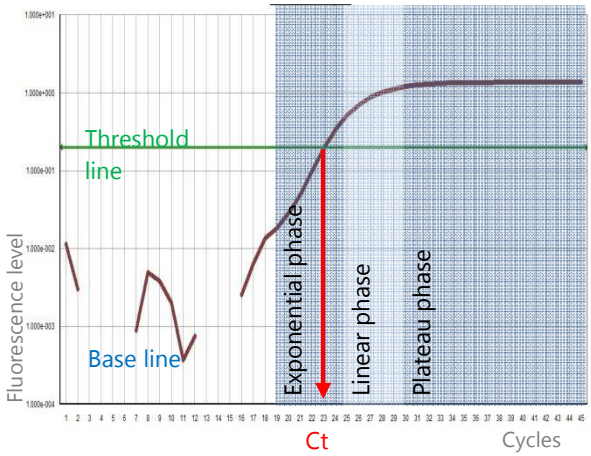
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present 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/HEX and Cy5 curves.

A. Validation of the run

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

Controls	No Template Control (NTC)	Amplification positive control (MS CTL+)	Amplification positive control (MG CTL+)	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	no	yes
Cy5 amplification	no	no	yes	no	yes
VIC/HEX amplification	yes	yes	yes	yes	yes/no
Validation of	Absence of contamination for amplification	Amplification of the target *M. synoviae*	Amplification of the target *M. gallisepticum*	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM, Cy5 and VIC/HEX dyes) of the CTL+ are indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are **valid** if at least a characteristic amplification curve is observed for *M. synoviae* (FAM), for *M. gallisepticum* (Cy5) or for the internal control (VIC/HEX).

Example	A	B	C	D	E
FAM amplification	No	Yes	No	Yes	No
Cy5 amplification	No	No	Yes	Yes	No
VIC/HEX amplification	Yes	Yes/No	Yes/No	Yes/No	No
Results	Negative	Positive for *M. synoviae*	Positive for *M. gallisepticum*	Positive for *M. synoviae* and *M. gallisepticum*	Undetermined

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

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (examples B and D) and/or in Cy5 (examples C and D). Internal control can be co-amplified.

A total absence of characteristic amplification curve for a sample (example E) 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.

VII. References

Yamamoto (1991). Mycoplasma meleagridis infection, In : B. W. Calnek, C. W. Beard, H. J. Barnes, M.
W. Reld and H. W. Yolder, J. R. Eds, Diseases of poultry, 9th ed. Iowa. State. University Press, Ames.
Iowa, p. 212-223.

Stipkovits L. and I. Kempf (1996). Mycoplasmosis in poultry. Rev Sci Tech. 15: 1495-1525

Kempf I. (1997). DNA amplification methods for diagnosis and epidemiological investigations of avian
mycoplasmosis. Acta vet Hung. 45: 373-386.

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

S.A.R.L. ADIAGENE
9, rue Gabriel Calloët-Kerbrat
22440 Ploufragan - France

RCS 417 876 299
Tel. +33 (0)2 96 68 40 20
www.biox.com