# ADIAVET™ MYCO AV FAST TIME

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

**References:**
ADI016-100 (100 reactions)
ADI016-500 (500 reactions)

VET

English version
NE016-01
2017/08

# ADIAVET™ MYCO AV FAST TIME

## Revision historic

| | |
|---|---|
| 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 historic

| Release Date | Part Number | Change type | Change summary |
|---|---|---|---|
| 2017/08 | NE016-01 | N/A | First publication |

# I. General informations

## 1. Purpose of the test

ADIAVET™ MYCO AV FAST 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).
All lot of real time PCR tests have been described providing specific and sensitive tools for avian mycoplasma detection (Kempf, 1997; Lauerman, 1998; Garcia et al., 2005, Sprygin et al., 2010).

## 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 FAST TIME 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 (Adiagene, 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 ADI016-100** | | |
| A5 | Amplification Solution | 1 x 1000 µl tubes green caps (ready to use) |
| MS CTL+ | Positive control *M. synoviae* | 1 tube purple caps (deshydrated) |
| MG CTL+ | Positive control *M. gallisepticum* | 1 tube purple caps (deshydrated)) |
| EPC-Amp | exogenous internal control of amplification | 1 x 150 µl tube white caps (ready to use) |
| **REF ADI016-500** | | |
| A5 | Amplification Solution | 5 x 1000 µl tubes green caps (ready to use) |
| MS CTL+ | Positive control *M. synoviae* | 1 tube purple caps (deshydrated) |
| MG CTL+ | Positive control *M. gallisepticum* | 1 tube purple caps (deshydrated)) |
| EPC-Amp | exogenous internal control of amplification | 2 x 150 µl tubes white caps (ready to use) |

## 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 Nuclease-free 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 a well.

## 4. Use of MG CTL+

MG CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free 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 a well.

## 5. Used of EPC-Amp

The EPC-Amp should only be used if an extraction method other than ADIAPURE™ SLB is used, for example after extraction by silica column extraction or ADIAPURE™ PURIFICATION.
Aliquot this solution and store it to <-15°C.
For each PCR reaction, mix 0.5 µl of EPC-Amp and 10 µl of « A5 solution ».
Do not defreeze each aliquot more than 3 times.

## 6. 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
- Nuclease-free water

- Peptone water
- Sterile saline water (NaCl 8.5 g/l)

**- DNA Extraction without purification:**

- ADIAPURE™ SLB (Bio-X Diagnostics; 500 ml : ref. ADIADP01S1-500)

**or**

**- 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)

**or**

**- DNA extraction kit (96-plate columns)**

- ADIAPURE™ PURIFICATION (Bio-X Diagnotics, 192 tests: ref. ADIADP001-192 or 480 tests: ref. ADIADP001-480)

## 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 Adiagene, 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. Controls preparation

The use of controls allows to verify 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 exogenous internal control of amplification added in A5 reagent verifies the amplification steps of each sample.
- The exogenous internal control of extraction added in all samples verifies the extraction and 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. Extractions et purifications

## 1. Extraction with ADIAPURE™ SLB kit

### a- Reagents

| REF ADIADP01S1-500 | | |
|---|---|---|
| L1 | lysis buffer | 5 x 100 ml bottles (ready-to-use) |
| L3 | lysis buffer | 1 x 25 ml bottle (ready-to-use) |

On receipt, the L3 buffer should be aliquoted and stored at **+2/8°C** or at -<15°C**.**

⚠ **Mix the buffer before use.**

**The L1 buffer should be placed at room temperature** in the darkness.
The L1 buffer can contains aggregate, warm it to obtain a clear solution before use.
**Do not mix reagents of two different batches.**

### b- Protocol

Put **1 to 3 swabs** in a microtube and add **1 ml of L1** buffer
Or
Put **4 to 6 swabs** in a microtube and add **2 ml of L1** buffer
Mix by vortexing 10 sec

*The swabs can be kept in L1 buffer at room temperature for 48h, for a longer conservation store at <-15°C.*

Transfer **50 µl** of the surpernatant in a microtube or in a well of PCR microplate, containing **50 µl of L3** buffer, **beforehand mixed**.
Mix by vortexing or pipetting.
Put an adhesive film if you used a microplate.

Incubate **15 minutes at +95°C** in heat block or thermal cycler.
Let to cool, to ensure the accuracy of subsequent pipetting.

*The solution can be kept at +2/8°C - 48 hours, for a longer conservation store at <-15°C.*

Then go to amplification §V.

## 2. Extraction using QIAamp® DNA Mini kit

### a) Preparation of samples

#### 1. swabs

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. 2b).
*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 by vortexing
Press the swab against the rim of the microtube to collect as much buffer as possible.
See § IV 2b 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 2b for the extraction and purification of DNA.
*NB: swabs in physiological water can be stored at +2/8°C.*

#### 2. 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. 2b).
Vortexing ~5 seconds.
Press the swab against the rim of the microtube to collect as much buffer as possible.
See § IV 2b for the extraction and purification of DNA.

#### 3. From liquid culture

Centrifuge (20 minutes at 10 000 g) **100** to **500 µl** of **culture** in a microtube.
Discard the supernatant.
See § IV 2b for the extraction and purification of DNA.

#### 4. 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 2b for the extraction and purification of DNA.

All the centrifugations are performed at room temperature.

**b) protocol**

| | Swab | Bacterial culture | Drag swabs |
|---|---|---|---|
| **Preparation of the sample** | See § IV.2.a | | |
| **Lysis** | Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Vortex.<br>Incubate **15 minutes** at **+56°C**. | | |
| | Add **200 µl** of **AL buffer**. Vortex.<br>Incubate **10 minutes** at **+70°C**. | | |
| **Binding preparation** | Add **200 µl** of **ethanol 100%**.<br>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.<br>Centrifuge 1 minute at 10 000 g.<br>*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.<br>Centrifuge 1 minute at 10 000 g. | | |
| **2nd wash** | Change the collection tube and add **500 µl** of **AW2 buffer** to the column.<br>Centrifuge 1 minute at 10 000 g. | | |
| **Column dry step** | Change the collection tube.<br>Centrifuge 3 minutes at 10 000 g. | | |
| **Elution** | Transfer the column to a microtube. Add **200 µl** of **AE buffer**.<br>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. | | |

## 3. Extraction using ADIAPURE™ PURIFICATION kit

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

## Amplification

a - Determine the *n* 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.

**Extraction with ADIAPURE™ SLB :**
Place **10 µl** of A5 solution in each PCR tubes or PCR plate wells.

**Other extraction:**
Place (n+1)***10 µl** of A5 solution in microtube,
Add (n+1)***0,5 µl « EPC-Amp »**.
Distribute **10 µl** of the solution in each PCR tubes or PCR plate wells

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 programs are defined for **ABI Prism** thermalcyclers (like 7500, StepOne...) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **MX3005P** and **ARIAMX** of **Agilent** and for **CFX96** of **BioRad**.

<table>
<tr><th colspan="2">Standard Program</th><th colspan="2">Short program</th></tr>
<tr><td colspan="2">2 min 50°C<br>10 min 95°C</td><td colspan="2">2 min. 95°C</td></tr>
<tr><td>15 sec</td><td rowspan="2">45 cycles</td><td>5 sec 95°C</td><td rowspan="2">45 cycles</td></tr>
<tr><td>1 min</td><td>30 sec 60°C</td></tr>
</table>

Contact us if you wish to use other thermalcyclers.

## 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.

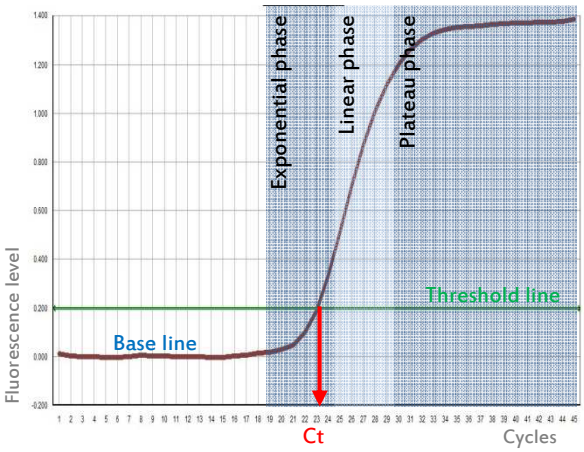
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

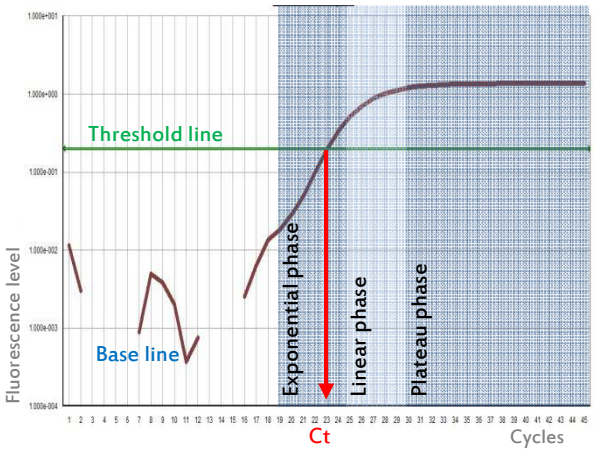
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present 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 |
| Amplification VIC/HEX | if EPC-Amp added in mix | no | no | no | yes | yes |
| | If EPC-Amp not added in mix | yes | yes | yes | yes | yes |
| Validation of | | Absence of contamination for amplification | Amplification of *M. synoviae* target | Amplification of *M. gallisepticum target* | 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.

## Literature references

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## 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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