INSTRUCTION MANUAL

ADIAVET™ MS-H DIVA FAST TIME

TEST FOR THE DETECTION AND DIFFERENTIATION OF *MYCOPLASMA SYNOVIAE* FIELD STRAINS FROM VACCINE STRAIN MS-H BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI561-100 (100 reactions)

VET

English version
NE561-01
2018-11

ADIAVET™ MS-H DIVA 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
2018-11	NE561-01	N/A	First publication

I. General informations

1. Purpose of the test

ADIAVET™ MS-H DIVA FAST TIME kit is intended to detect and to differentiate *Mycoplasma synoviae* field strains from vaccine strain MS-H using real-time Polymerase Chain Reaction (PCR) technology from swab of poultry.

2. 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™ MS-H DIVA FAST TIME kit enables the simultaneous detection of:

- *M. synoviae* field strains (probe labelled in FAM),
- Vaccine strain MS-H (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).

Adiagene recommends using this test with DNA purification kits (Adiagene, Qiagen). 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
Bacterial culture (solid, liquid)	☑	☒
FTA card	☑	☒

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

II. Material & reagents

1. Reagents provided with the kit

REF ADI561-100		
A5	Amplification Solution	2 x 500 µl tubes green caps (ready to use)
MS CTL+	Positive control *M. synoviae* field strain	1 tube purple caps (dehydrated)
MS-H CTL+	Positive control vaccine strain MS-H	1 tube purple caps (dehydrated))
EPC-Amp	exogenous internal control of amplification	1 x 150 µl tube 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-H CTL+

MS-H CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free water to the MS-H 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-H CTL+ in a well.

4. 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 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 MS 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.
Aliquot this solution and store it to <-15°C. **Do not defreeze each aliquot more than 3 times.**
For each PCR reaction, mix **0.5 µl of EPC-Amp and 10 µl of « A5 solution ».**

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
- 2% Peptone water
- Sterile saline water (NaCl 8.5 g/l)

- **DNA Extraction without purification:**
  - ADIAPURE™ SLB (Bio-X Diagnostics; 500 ml: ref. ADIADP01S1-500)
  - ADIAPURE™ SLB (Bio-X Diagnostics; 100 ml: ref. ADIADP01S1-100)

or

- **DNA extraction kit (individual silica columns)**
  - QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)

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 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 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 (EPC-Amp) added in A5 reagent verifies the amplification steps of each sample. (if using an extraction kit other that the ADIAPURE SLB kit)
- The exogenous internal control of extraction (included in the L3 buffer in ADIAPURE SLB extraction kit) added in all samples verifies the extraction and amplification steps of each sample.
- The CTL+ validate the amplification of the both targets.

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 could be a negative sample or a buffer used for dilutions (sterile saline water or PBS 1X for example).

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

A positive control could be added in each trial. The control is a sample including *M. synoviae* field strain and/or vaccine strain MS-H. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *M. synoviae* field strain and/or vaccine strain MS-H. This positive control should 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 ADIADP01S1-100		
L1	lysis buffer	1 x 100 ml bottle (ready-to-use)
L3	lysis buffer	1 x 5 ml bottle (ready-to-use)
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 each 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 seconds each tube

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

Transfer **50 µl** of the surpernatant in a microtube or in a well of PCR microplate, containing **50 µl of L3** buffer, **previously 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

Note: It is best to treat heavily soiled autopsy swabs individually.

1st method

Put **1 swab** or vortex successively **up to 3 swabs** in a microtube containing **180 µl** of **ATL buffer** and **20 µl** of **proteinase K.**

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 **saline water**.

Vortex ~10 seconds.

Transfer **200 µl** of **supernatant** in a microtube containing **180 µl** of **ATL buffer** and **20 µl** of **proteinase K**.

Mix by vortexing

See § IV 2b for the extraction and purification of DNA.

NB: swabs in saline 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 **180 µl** of **ATL buffer** and **20 µl** of **proteinase K.**

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.

Add **180 µl** of **ATL buffer** and **20 µl** of **proteinase K.**

Mix by vortexing

See § IV 2b for the extraction and purification of DNA.

4. From FTA card

Cut the 1 cm² of FTA card and intriduice its in a microtube

Add **1 ml** of saline water

Incube overnight at room temperature

Tranfert **200 µl** of supernatant in a microtube containing **180 µl** of **ATL buffer** and **20 µl** of **proteinase K**.

Mix by vortexing

See § IV 2b for the extraction and purification of DNA.

b) protocol

All the centrifugations are performed at room temperature.

	Swab / Bacterial culture / FTA card
Preparation of the sample	See § IV.2.a
Lysis	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.

V. 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 and prepare the amplification solution A5 :

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 10 µl of A5 solution.
For the CTL+, add **5 µl** of the solution obtained in § II-3 or § II-4 to the 10 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 as soon as possible.

The *M. synoviae* field strain target is read in FAM. The vaccine strain MS-H 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 (at 60°C).

The following programs are defined for **ABI Prism** thermalcyclers (like 7500, StepOne, QS5...) 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
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>

* Note 32 secondes for the ABI7500 thermofisher

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.

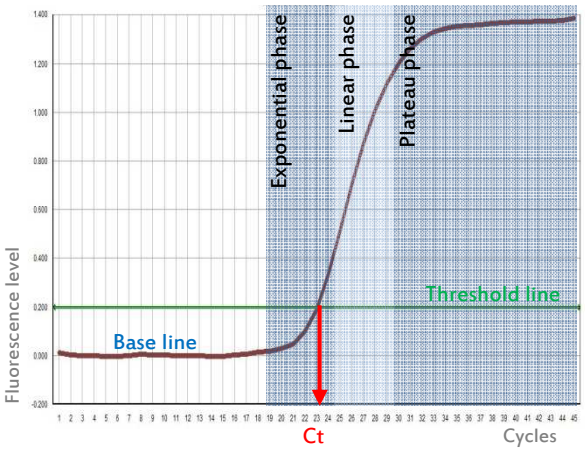
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

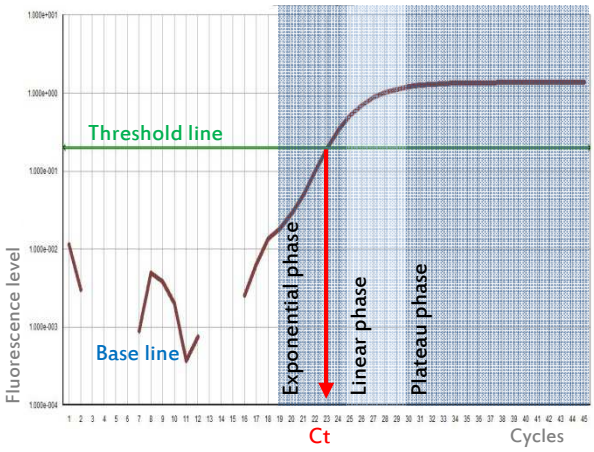
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

Example of characteristic amplification curve :

Linear scale of Y axis



Logarithmic scale of Y axis

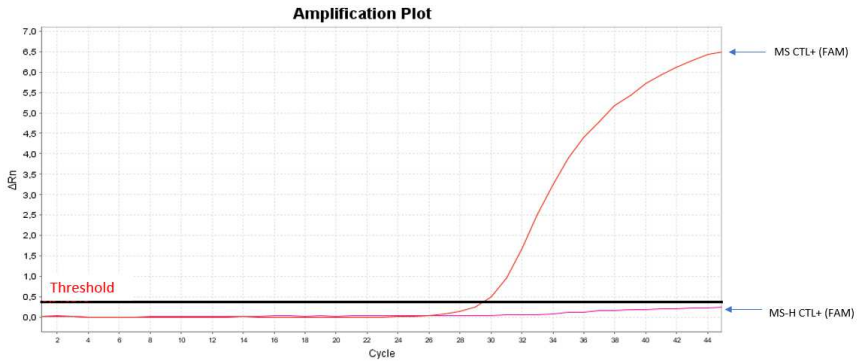


2. Validation and interpretation of results

a. Threshold line position

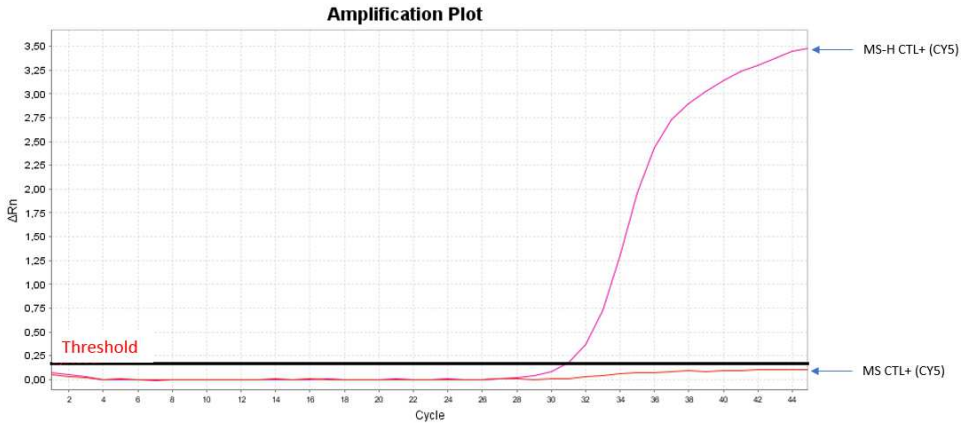
Display the FAM curves obtained with the "MS CTL+" and "MS-H CTL+".
The threshold line must be positioned manualy. In FAM linear scale, a strong inscrease in fluorescence of "MS CTL+" is expected. With some thermalcyclers, it's possible to observe a fluorescence near to the background with the "MS-H CTL+". In this case, position the threshold line just above the background fluorescence of "MS-H CTL+" like the following example.

<u>Example with the 7500 thermacycler in FAM:</u>



Display the Cy5 curves obtained with the "MS CTL+" and "MS-H CTL+".
The threshold line must be positioned manualy. In Cy5 linear scale, a strong inscrease in fluorescence of "MS-H CTL+" is expected. With some thermalcyclers, it's possible to observe a fluorescence near to the background with the "MS CTL+". In this case, position the threshold line just above the background fluorescence of "MS CTL+" like the following example.

Example with the 7500 thermacycler in Cy5:



b. Validation of the run

Display the obtained curves in FAM, Cy5 and VIC/HEX to analyze the results.
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 (MS-H 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* field strain target	Amplification of vaccine strain MS-H 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 mentioned in the certificate of analysis provided with the kit.

c. Result interpretation

DNA extraction and amplification for each sample are **valid** if at least a characteristic amplification curve is observed for *M. synoviae* field strain (FAM), for vaccine strain MS-H (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	No detected	Detected for *M. synoviae* field strain	Detected for vaccine strain MS-H	Detected for *M. synoviae* field strain and vaccine strain MS-H	Undetermined

The sample is considered as **No detected** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (example A).

The sample is considered as **Detected** 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 (loss 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. 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